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TOXICOLOGICAL REVIEW

OF

CHLOROFORM

(CAS No. 67-66-3)

In Support of Summary Information on the Integrated Risk Information System (IRIS)

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U.S. Environmental Protection Agency
Washington, DC

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ACRONYM LIST

AIC	Akaike information criterion
ATP	Adenosine tri-phosphate
BDCM	Bromodichloromethane
BMD	Benchmark dose
BMDL	A lower one-sided confidence limit on the BMD
BMDS	Benchmark dose software
BMR	Benchmark response
BrdU	Bromodeoxyuridine
CHO	Chinese hamster ovary
CYP2E1	Cytochrome P-450-2E1
DEN	Diethylnitrosamine
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
GGT	Gamma glutamyl transferase
GOT	Glutamate oxaloacetate transaminase (aspartate aminotransferase)
ICPEMC	International Commission for Protection against Environmental Mutagens
ILSI	International Life Sciences Institute
IRIS	Integrated Risk Information System
LD50	Lethal Dose 50 (dose causing death in 50% of the exposed animals)
LDH	Lactate dehydrogenase
LI	Labeling index
LOAEL	Lowest-observed-adverse-effect-level
NCI	National Cancer Institute
NOAEL	No-observed-adverse-effect-level
PBPK	Physiologically based pharmacokinetic models
ppm	Parts per million
RBC	Red blood cell
RfD	Reference dose
SAP	Serum alkaline phosphatase
SGPT	Serum glutamate pyruvate transaminase (alanine aminotransferase)
THM	Trihalomethane
TTHM	Total trihalomethanes
U.S. EPA	United States Environmental Protection Agency

FOREWORD

The purpose of this Toxicological Review is to provide scientific support and rationale for the hazard and dose-response assessments in IRIS pertaining to chronic exposure to chloroform. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of chloroform.

In Section 6, EPA has characterized its overall confidence in the quantitative and qualitative aspects of hazard and dose response. Matters considered in this characterization include knowledge gaps, uncertainties, quality of data, and scientific controversies. This characterization is presented in an effort to make apparent the limitations of the assessment and to aid and guide the risk assessor in the ensuing steps of the risk assessment process.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to EPA's IRIS Hotline at 301-345-2870.

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Reviews

This Toxicological Review of Chloroform was based in part on the Health Risk Assessment/Characterization of the Drinking Water Disinfectant Byproduct Chloroform and the Draft Chloroform Risk Assessment (mode-of-action analysis for the carcinogenicity of chloroform). Both documents have been peer-reviewed. The mode-of-action analysis was reviewed by the Agency's Science Advisory Board (SAB) in October 1999. The SAB reviewers and consultants are listed below, and the SAB report can be found on the web at *http://www.epa.gov/sab/fiscal00.htm.* The Agency response to SAB comments is shown following the names of SAB reviewers. The Health Risk Assessment/Characterization of the Drinking Water Disinfectant Byproduct Chloroform is peer-reviewed both by EPA scientists (see Internal EPA Reviewers) and by independent scientists external to EPA (see External Peer Reviewers). Summaries of the external peer reviewers' comments and the disposition of their recommendations are in Appendix A. Subsequent to the external review and incorporation of comments, this Toxicological Review of Chloroform and IRIS Summaries have been written and undergone an Agencywide review process whereby the IRIS program manager has achieved a consensus approval among the Office of Research and Development; Office of Air and Radiation; Office of Prevention, Pesticides, and Toxic Substances; Office of Solid Waste and Emergency Response; Office of Water; Office of Policy; Office of Children's Health Protection; and the Regional Offices.

Before the reviews mentioned above, International Life Sciences Institute (ILSI) provided a formal review of chloroform mode of action as part of a cooperative agreement with EPA. A panel of ten scientific experts reviewed the literature and issued a report on the carcinogen risk assessment of chloroform in November 1997. Similar to the SAB report, the ILSI report supported a nonlinear approach for risk estimation.

As recommended by the SAB, a systematic analysis of the genotoxicity of chloroform, including the most recent in vivo and in vitro studies, is included in this document and in the IRIS summaries. A brief discussion of the epidemiological studies of chlorinated drinking water (a mixture of disinfection byproducts including chloroform) is also included in this document. On the noncancer endpoint, a more complete RfD analysis is performed including the traditional NOAEL/LOAEL and the benchmark dose approaches. The final value is coincidentally the same as the one previously on IRIS.

Internal EPA Reviewers

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External Peer Reviewers and Affiliation

External peer reviewers who provided comments on EPA's evaluation of chloroform are listed below:

James A. Swenberg, D.V.M., Ph.D., University of North Carolina
Lorenz Rhomberg, Ph.D., Gradient Corporation
Sandra Baird, The Baird Group
R. Julian Preston, Ph.D., Chemical Industry Institute of Toxicology

Summaries of the external peer reviewers' comments and the disposition of their recommendations are presented in Appendix A.

SAB Review of the Mode of Action of Chloroform

Co-chairs, members, and consultants of the SAB who provided review comments on EPA's evaluation of chloroform are listed below:

Dr. Richard J. Bull, Battelle Pacific Northwest National Laboratory (Co-chair)
Dr. Mark J. Utell, University of Rochester Medical Center (Co-chair)

Dr. Mary Davis, West Virginia University (member)
Dr. George Lambert, Robertwood Johnson University (member)
Dr. Lauren Zeise, California Environmental Protection Agency (member)
Dr. James E. Klaunig, Indiana University School of Medicine (consultant)
Dr. Richard Okita, Washington State University (consultant)
Dr. David Savitz, University of North Carolina, School of Public Health (consultant)
Dr. Verne Ray, Toxicologist (consultant)
Dr. Robert Maronpot, NIEHS (Federal Expert)

A summary of the comments provided by the SAB and EPA's response to those comments is presented in the following two pages.

SUMMARY OF SCIENCE ADVISORY BOARD RECOMMENDATIONS AND EPA RESPONSES

In October 1999 the Chloroform Risk Assessment Review Subcommittee of the Science Advisory Board met to consider the Office of Science and Technology health assessment of chloroform. Summaries of the major parts of the subcommittee's advice and our responses follow. The documents reviewed were a final hazard and dose-response characterization document and a draft mode-of-action framework analysis.

1. The subcommittee agreed with EPA that sustained or repeated cytotoxicity with secondary regenerative hyperplasia in the liver and/or the kidney of rats and mice precedes, and is probably a causal factor for, hepatic and renal neoplasia**.** Some members of the subcommittee were concerned about possible mutagenic activity, and the subcommittee recommended that the risk assessment further address the possible role of mutagenicity as a mode of action.

   EPA Response: The Office of Water has included a more complete analysis of mutagenic potential in the final Toxicological Review of Chloroform.

2. The Subcommittee concluded that a nonlinear margin-of-exposure approach is scientifically reasonable for the liver tumor response because of the strong role cytotoxicity appears to play. In contrast, the application of the standard linear approach to the liver tumor data is likely to substantially overstate the low-dose risk. In addition, there is considerable question about this response because it is not produced when chloroform is administered to mice in drinking water.

   For the kidney response, because sustained cytotoxicity plays a clear role in the rat, a margin of exposure (MOE) is a scientifically reasonable approach. Most members of the subcommittee thought that genotoxicity might possibly contribute to low-dose response in this organ, while some thought it unlikely.

   EPA Response: The Office of Water has utilized the MOE approach recommended by SAB, but has also noted the reservations of some committee members regarding a potential role for genotoxicity.

3. The subcommittee concluded that the extensive epidemiologic evidence relating drinking water disinfection (specifically chlorination) with cancer has little bearing on the determination of whether chloroform is a carcinogen. It added recommendations for discussion of endpoints and the potential meaning of these data to the assessment of chloroform.

   EPA Response: The hazard and dose-response assessment document reviewed by SAB did not contain the complete analysis of epidemiologic studies and the population-attributable risk analysis. The latter were separately provided to the subcommittee. The

Toxicological Review for Chloroform does provide a summary of these studies along with a discussion of their limitations in evaluating cancer risk from chloroform in humans.

4. The subcommittee found that the draft mode-of-action document addressed children's risks quite adequately, based on the scientific information currently available. The major conclusions were believed correct, the role of CYP2E1 should be expressed as important, and its definitive role in the developing human or (other) mammal has yet to be confirmed. Nevertheless, the subcommittee report discussed knowledge of children's potential risk in several areas, such as exposure latency and transplacental and transmamillary exposure, that can be improved.

   EPA Response: The Office of Water has revised the Toxicological Review in accord with the SAB recommendations. As the advice on some issues appears to be applicable beyond the chloroform assessment and to carry implications for Agency guidance documents, the advice will be discussed with the EPA Risk Assessment Forum.

1. INTRODUCTION

This document presents background and justification for the hazard and dose-response assessment summaries in EPA's Integrated Risk Information System (IRIS). IRIS summaries may include an oral reference dose (RfD), inhalation reference concentration (RfC), and a carcinogenicity assessment.

The RfD and RfC provide quantitative information for noncancer dose-response assessments. The RfD is based on the assumption that thresholds exist for certain toxic effects such as cellular necrosis but may not exist for other toxic effects such as some carcinogenic responses. It is expressed in units of mg/kg/day. In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The inhalation RfC is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extrarespiratory or systemic effects). It is generally expressed in units of mg/m^3.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral exposure and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are presented in three ways. The *slope factor* is the result of application of a low-dose extrapolation procedure and is presented as the risk per mg/kg/day. The *unit risk* is the quantitative estimate in terms of either risk per µg/L drinking water or risk per $µg/m^3$ air breathed. Another form in which risk is presented is as a drinking water or air concentration providing cancer risks of 1 in 10,000; 1 in 100,000; or 1 in 1,000,000.

Development of these hazard identification and dose-response assessments for chloroform has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). EPA guidelines that were used in the development of this assessment may include the following: the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 1986a), *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986b), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986c), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Proposed Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Proposed Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 1996a), *Draft Revisions of the Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 1999), *Reproductive Toxicity Risk Assessment Guidelines* (U.S. EPA, 1996b); *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988); (proposed) *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity* (U.S. EPA, 1994a); *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b); *Peer Review and Peer Involvement at the U.S. Environmental Protection Agency* (U.S. EPA, 1994c); *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995a); *Science Policy Council Handbook: Peer Review* (U.S. EPA, 1998b); and memorandum from EPA Administrator, Carol Browner, dated March 21, 1995, Subject: Guidance on Risk Characterization.

The literature search strategies employed for this compound were based on the CASRN and at least one common name. At a minimum, the following databases were searched: RTECS, HSDB, TSCATS, CCRIS, GENETOX, EMIC, EMICBACK, DART, ETICBACK, TOXLINE, CANCERLINE, MEDLINE, and MEDLINE backfiles. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document.

2. CHEMICAL AND PHYSICAL INFORMATION

Chloroform (trichloromethane) is a colorless, volatile liquid with a distinct odor. Chloroform is nonflammable. It is slightly soluble in water and is readily miscible with most organic solvents (Lewis 1993). Selected chemical and physical properties of chloroform are listed below (Howard and Meylan 1997):

CASRN:	67-66-3
Empirical formula:	$CHCl_3$
Molecular weight:	119.38
Density:	1.483 g/mL
Vapor pressure:	197 mm Hg at 25°C
Henry's Law Constant:	3E-03 atm-m^3/mole (0.12 mg/L in air per mg/L in water)
Water solubility:	7.95 g/L at 25°C
Log K_{ow}:	1.97
Conversion factor (air):	1 ppm = 4.88 mg/m^3
	1 mg/m^3 = 0.205 ppm

Because chloroform is relatively volatile, it tends to escape from contaminated environmental media (e.g., water or soil) into air, and may also be released in vapor form from some types of industrial or chemical operations. Therefore, humans may be exposed to chloroform not only by ingestion of chloroform in drinking water, food, or soil, but also by dermal contact with contaminated media (especially water) and by inhalation of vapor (especially in indoor air).

3. TOXICOKINETICS

3.1. ABSORPTION

Studies in animals indicate that gastrointestinal absorption of chloroform is rapid (peak blood levels at about 1 hour) and extensive (64% to 98%) (U.S. EPA, 1997; ILSI, 1997; U.S. EPA, 1998c). Limited data indicate that gastrointestinal absorption of chloroform is also rapid and extensive in humans, with more than 90% of an oral dose recovered in expired air (either as unchanged chloroform or carbon dioxide) within 8 hours (Fry et al., 1972).

Most studies of chloroform absorption following oral exposure have used oil-based vehicles and gavage dosing (U.S. EPA, 1994d, 1998c). This is of potential significance because most humans are exposed to chloroform by ingestion in drinking water. Withey et al. (1983) compared the rate and extent of gastrointestinal absorption of chloroform following gavage administration in either aqueous or corn oil vehicles. Twelve male Wistar rats were administered single oral doses of 75 mg chloroform/kg via gavage. The time-to-peak blood concentration of chloroform was similar for both vehicles; however, the concentration of chloroform in the blood was lower at all time points for the animals administered chloroform in the oil vehicle compared with animals administered the water vehicle. The authors interpreted this to indicate that the rate of chloroform absorption was higher from water than from oil, although differences in the rate of first-pass metabolism in the liver might contribute to the observed difference (U.S. EPA, 1994d, 1998c).

Dermal and inhalation absorption of chloroform by humans during showering was investigated by Jo et al. (1990). Chloroform concentrations in exhaled breath were measured in six human subjects before and after a normal shower, and following inhalation-only shower exposure. Breath levels measured at 5 minutes following either exposure correlated with tap water levels of chloroform. Breath levels following inhalation exposure only were about half those following a normal shower (both inhalation and dermal contact). These data indicate that humans absorb chloroform by both the dermal and inhalation routes (U.S. EPA, 1994d).

3.2. DISTRIBUTION

Absorbed chloroform appears to distribute widely throughout the body (U.S. EPA, 1994d, 1998c). In postmortem samples from eight humans, the highest levels of chloroform were detected in the body fat (5–68 ：g/kg), with lower levels (1–10 ：g/kg) detected in the kidney, liver, and brain (McConnell et al., 1975). Studies in animals indicate rapid uptake of chloroform by the liver and kidney (U.S. EPA, 1997). In mice receiving chloroform via gavage in either corn oil or water, maximal uptake of chloroform was achieved within 10 minutes in the liver and within 1 hour in the kidney (Pereira, 1994). Following intraperitoneal injection of 150 mg/kg ^{14}C-chloroform, peak radioactivity levels were achieved in the liver, kidney, and blood of male mice within 10 minutes of dosing, and had returned to background levels within 3 hours (Gemma et al., 1996).

3.3. METABOLISM

3.3.1. Oxidative and Reductive Pathways

Chloroform is metabolized in humans and animals by cytochrome P450-dependent pathways. In the presence of oxygen (oxidative metabolism), the chief product is trichloromethanol, which rapidly and spontaneously dehydrochlorinates to form phosgene (CCl_2O):

$$2\ CHCl_3 + NADPH + H^+ + O_2 \quad 2\ CCl_3OH + NADP^+$$
$$CCl_3OH \quad CCl_2O + HCl$$

In the absence of oxygen (reductive metabolism), the chief metabolite is dichloromethyl free radical ($CHCl_2$) (U.S. EPA, 1997; ILSI, 1997).

Nearly all tissues of the body are capable of metabolizing chloroform, but the rate of metabolism is greatest in liver, kidney cortex, and nasal mucosa (ILSI, 1997). These tissues are also the principal sites of chloroform toxicity, indicating the importance of metabolism in the mode of action of chloroform toxicity.

At low chloroform concentrations, metabolism occurs primarily via cytochrome P450-2E1 (CYP2E1) (Constan et al., 1999). The level of this isozyme (and hence the rate of chloroform metabolism) is induced by a variety of alcohols (including ethanol) and ketones, and may be inhibited by phenobarbital. At high chloroform concentrations, metabolism is also catalyzed by cytochrome P450-2B1/2 (CYP2B1/2) (ILSI, 1997; U.S. EPA, 1997, 1998c). Because chloroform metabolism is enzyme-dependent, the rate of metabolism displays saturation kinetics. Under low dose-rate conditions, nearly all of a dose is metabolized. However, as the dose or the dose rate increases, metabolic capacity may become saturated and increasing fractions of the dose are excreted as the unmetabolized parent (Fry et al., 1972).

3.3.2. Fate of Reactive Metabolites

The products of oxidative metabolism (phosgene) and reductive metabolism (dichloromethyl free radical) are both highly reactive. Phosgene is electrophilic and undergoes attack by a variety of nucleophiles. The predominant reaction is hydrolysis by water, yielding carbon dioxide and hydrochloric acid:

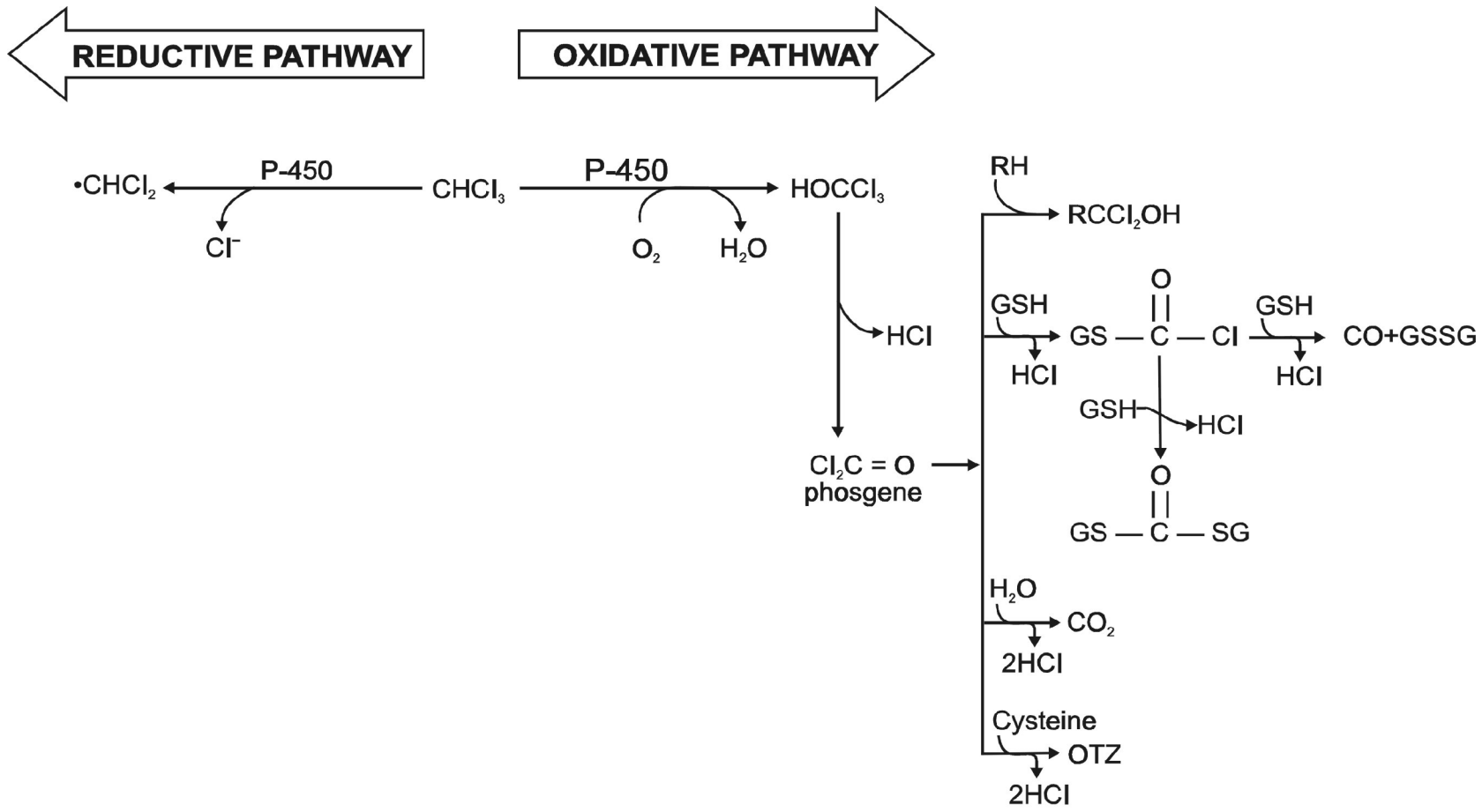
$$CCl_2O + H_2O \rightarrow CO_2 + 2\ HCl$$

The rate of phosgene hydrolysis is very rapid, with a half-time of less than 1 second (De Bruyn et al., 1995). Phosgene also reacts with a wide variety of other nucleophiles, including primary and secondary amines, hydroxy groups, and thiols (Schneider and Diller, 1991). For example, phosgene reacts with the thiol group of glutathione (GSH), yielding S-chloro-carbonyl glutathione, which in turn can either interact further with glutathione to form diglutathionyl dithiocarbonate, or form glutathione disulfide and carbon monoxide (ILSI, 1997):

$$CCl_2O + GSH \rightarrow GSCOCl + HCl$$

$$GSCOCl + GSH \rightarrow GS\text{-}CO\text{-}SG + HCl$$
$$GSCOCl + GSH \rightarrow GSSG + CO + HCl$$

Phosgene also undergoes attack by nucleophilic groups (-SH, -OH, $-NH_2$) in cellular macromolecules such as enzymes, proteins, or the polar heads of phospholipids, resulting in formation of covalent adducts (Pohl et al., 1977, 1980, 1981; Pereira and Chang, 1981; Pereira et al., 1984; Noort et al., 2000). Formation of these molecular adducts can interfere with molecular function (e.g., loss of enzyme activity), which in turn may lead to loss of cellular function and subsequent cell death (ILSI, 1997; WHO, 1998).



R = cellular nucleophile (protein, phospholipid, nucleic acid); GSH = reduced glutathione; GSSG = oxidized glutathione; OTZ = oxothiazolidine carboxylic acid; P-450 = cytochrome P-450

Source: Adapted from Stevens and Anders (1981), Tomasi et al. (1985), and ILSI (1997).

FIGURE 1. METABOLIC PATHWAYS OF CHLOROFORM BIOTRANSFORMATION

Free radicals that are formed under conditions of low oxygen are also extremely reactive, forming covalent adducts with microsomal enzymes and the fatty acid tails of phospholipids, probably quite close to the site of free radical formation (cytochrome P450 in microsomal membranes). This results in a general loss of microsomal enzyme activity, and can also result in lipid peroxidation (ILSI, 1997; U.S. EPA, 1998c).

3.3.3. Relative Importance of Oxidative and Reductive Pathways

A priori, it might be expected that the oxidative pathway of chloroform metabolism would predominate in vivo, because tissues of healthy animals are oxygenated. However, some investigators have noted that the centrilobular region of the liver, where chloroform hepatotoxicity is largely localized, is physiologically hypoxic, with oxygen partial pressures from 0.1% to 8% (U.S. EPA, 1998c; ILSI, 1997).

Nevertheless, two lines of evidence suggest that metabolism occurs mainly via the oxidative pathway. First, reductive metabolism of chloroform is observed only in phenobarbital-induced animals or in tissues prepared from them, with negligible reducing activity observed in uninduced animals (ILSI, 1997). Second, in vitro studies using liver and kidney microsomes from mice indicate that, even under relatively low (2.6%) oxygen partial pressure (approximately average for the liver), more than 75% of the phospholipid binding was to the fatty acid heads. This pattern of adduct formation on phospholipids is consistent with phosgene, not free radicals, as the main reactive species, indicating metabolism was chiefly by the oxidative pathway (U.S. EPA, 1998c; ILSI, 1997). Addition of glutathione to the incubation system completely negated binding to liver microsomes, with only residual binding remaining in kidney microsomes (ILSI, 1997). This quenching by glutathione is expected for the products of oxidative but not reductive metabolism. Taken together, these observations strongly support the conclusion that chloroform metabolism in vivo occurs primarily via the oxidative pathway, except under special conditions of high chloroform doses in preinduced animals (ILSI 1997, U.S. EPA 1998c).

3.4. EXCRETION

Excretion of chloroform occurs primarily via the lungs (U.S. EPA, 1998c). Results from studies in humans indicate that approximately 90% of an oral dose of chloroform was exhaled (either as chloroform or as carbon dioxide), with less than 0.01% of the dose excreted in the urine (U.S. EPA, 1994d). In mice and rats, 45%–88% of an oral dose of chloroform was excreted from the lungs either as chloroform or carbon dioxide, with 1%–5% excreted in the urine (U.S. EPA, 1998c).

No data are available regarding the bioaccumulation or retention of chloroform following repeated exposure. However, because of the rapid excretion and metabolism of chloroform, combined with low levels of chloroform detected in human postmortem tissue samples, marked accumulation and retention of chloroform is not expected (U.S. EPA, 1994d).

3.5. PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELS

The concentration of a chemical that reaches a target tissue following some external exposure depends not only on the external dose administered to the organism (human or animal),

but also on a number of physiological parameters that may differ significantly from organism to organism. Likewise, the rate and extent of metabolism of the chemical to less toxic or more toxic intermediates may also vary from tissue to tissue and from organism to organism. Therefore, extrapolation of toxicological observations from dose to dose, from route to route, and from organism to organism are all quite uncertain unless a detailed understanding exists regarding the absorption, distribution, metabolism, and clearance of the chemical. Mathematical models that describe the rate and extent of absorption, distribution, metabolism, and clearance as a function of dose, time, route, and organism-specific physiological parameters are referred to as physiologically based pharmacokinetic (PBPK) models.

Corley et al. (1990) developed a PBPK model for chloroform. In brief, the model consists of a series of differential equations that describe the rate of chloroform entry into and exiting from each of a series of body compartments, including (1) gastrointestinal tract, (2) lungs, (3) arterial blood, (4) venous blood, (5) liver, (6) kidney, (7) other rapidly perfused tissues, (8) slowly perfused tissues, and (9) fat. In general, the rate of input to each compartment is described by the product of (a) the rate of blood flow to the compartment, (b) the concentration of chloroform in arterial blood, and (c) the partition coefficient between blood and tissue. Absorption of chloroform into the blood from the lungs or stomach is modeled by assuming first-order absorption kinetics. Material absorbed from the stomach is assumed to flow via the portal system directly to the liver (the "first-pass effect"), while material absorbed from the lungs enters the arterial blood. Each tissue compartment is assumed to be well mixed, with venous blood leaving the tissue being in equilibrium with the tissue. Metabolism of chloroform is assumed to occur in both the liver and the kidney. The rate of metabolism is assumed to be saturable and is described by Michaelis-Menten type equations. Chloroform metabolism is assumed to lead to binding of a fraction of the total metabolites to cellular macromolecules, and the amount bound is one indicator of the delivered dose. Binding of reactive metabolites to cell macromolecules is also assumed to cause a loss of some of the metabolic capacity of the cell. This metabolic capacity (enzyme level) is then resynthesized at a rate proportional to the amount of decrease from the normal level. Based on a review of published physiological and biochemical data, as well as several studies specifically designed to obtain model parameter estimates, Corley et al. (1990) provided recommended values for each of the model inputs for three organisms (mouse, rat, and human). These values are shown in Table 1. On the basis of these inputs, the model predicted that the amount of chloroform metabolized per unit dose per kg of tissue (liver or kidney) would be highest in the mouse, intermediate in the rat, and lowest in the human. This difference between species is due to the lower rates of metabolism, ventilation, and cardiac output in larger species compared to smaller species. If equal amounts of metabolite binding to cellular molecules were assumed to be equitoxic to tissues, then the relative potency of chloroform would be mice > rats > humans.

The model was extended by Reitz et al. (1990), who added equations describing the effect of chloroform metabolism on cell killing in the liver. It was assumed that cells were subject to risk of death when the rate of metabolism exceeded the ability of the cell to detoxify the metabolic products, with the probability of any particular cell dying being characterized by a normal distribution function. In addition, it was assumed that cell death did not occur instantly, but depended on both the rate of metabolism and the time of exposure. Results from this model

Table 1. Summary of PBPK parameters

Parameter	Tissue/compartment	Mouse	Rat	Human
Body weight (kg)	--	0.0285	0.230	70.0
Percentage of body weight	Liver	5.86	2.53	3.14
	Kidney	1.70	0.71	0.44
	Fat	6.00	6.30	23.1
	Rapidly perfused	3.30	4.39	3.27
	Slowly perfused	74.1	77.1	61.1
Flows (L/hr)	Alveolar ventilation	2.01	5.06	347.9
	Cardiac output	2.01	5.06	347.9
Tissue blood flow (% cardiac output)	Liver	25.0	25.0	25.0
	Kidney	25.0	25.0	25.0
	Fat	2.0	5.0	5.0
	Rapidly perfused	29.0	26.0	26.0
	Slowly perfused	19.0	19.0	19.0
Partition coefficients	Blood/air	21.3	20.8	7.43
	Liver/air	19.1	21.1	17.0
	Kidney/air	11.0	11.0	11.0
	Fat/air	242	203	280
	Rapidly perfused/air	19.1	21.1	17.0
	Slowly perfused/air	13.0	13.9	12.0
Metabolic constants	$V_{max}C$ (mg/kg/hr)	22.8	6.8	15.7
	K_m (mg/L)	0.352	0.543	0.448
	k_{loss} (L/mg)	5.72E-4	0	0
	k_{resysn} (1/hr)	0.125	0	0
	A (kidney/liver)	0.153	0.052	0.033
	fMMB in liver (1/hr)	0.003	0.00104	0.00202
	fMMB in kidney (1/hr)	0.010	0.0086	0.00931
Gastric absorption rate constants	k_{as} from corn oil (1/hr)	0.6	0.6	0.6
	k_{as} from water (1/hr)	5.0	5.0	5.0

All values are derived from Corley et al., 1990.

predicted that the number of cells killed depended on the dose route, with higher toxicity via gavage exposure than drinking water exposure. This supports the view that the hepatotoxicity of chloroform (and hence the potential for carcinogenicity) is strongly dependent on rate of metabolism, which in turn is dependent on dose rate.

The Corley model was adapted by Blancato and Chiu (1994) to include dermal exposure from water while bathing or swimming. The EPA model was validated by comparing results with those obtained by Corley et al. for identical input assumptions, and by comparing results for the same model established in a separate simulation environment (SIMUSOLVE). In both cases, model results were nearly identical for all cases compared, indicating that the model is mathematically valid. Blancato and Chiu (1994) applied the model to several human exposure scenarios where data were available on the amount of chloroform in exhaled air. The model predictions fit the data well, supporting the accuracy of the underlying model and the pharmacokinetic input values.

Smith et al. (1995) also adapted the basic Corley et al. (1990) model to evaluate the relative merits of various estimates of internal dose as predictors of rodent tumor bioassay data. These workers found that dose-rate-dependent measures (maximal rate of metabolism and percentage of hepatocytes killed per day) correlated well for the rodent liver bioassay data. In contrast, none of the model dose parameters predicted the kidney bioassay data as well as dose scaled to body surface area.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS

4.1.1. Inhalation Studies in the Workplace

A number of epidemiological studies have been performed to investigate the occurrence of adverse effects in populations of workers exposed to chloroform vapors in the workplace. In general, these studies must be interpreted cautiously, because data on actual chloroform exposure are generally lacking, and most workplace studies involved exposures to other chemicals besides chloroform.

Based on the limited data available, and subject to the cautions mentioned above, it appears that long term exposure to concentrations of 100-1,000 mg/m^3 (20-200 ppm) of chloroform produce mainly neurological effects, with increased incidence of symptoms such as fatigue, nausea, vomiting, lassitude, dry mouth, and anorexia (Phoon et al., 1983; Challen et al., 1958; Li et al., 1993; Bomski et al., 1967). Some studies also observed effects on the liver, including jaundice, increased serum enzyme levels, and increased liver size (Phoon et al., 1983; Bomski et al., 1967). Available data are not adequate to define with confidence the inhalation dose-response curve in humans for either neurological or hepatic effects, but data from Li et al. (1993) suggest hepatic effects are not likely at exposure concentrations of 30 mg/m^3 (6 ppm) or lower, and essentially no effects were detected at concentrations of about 13 mg/m^3 (2.6 ppm). An association between chloroform exposure and increased risk of spontaneous abortion was reported for workers in biomedical research laboratories (Wennborg et al., 2000), but no data on

actual exposure levels were presented, and the workers were also known to be exposed to numerous other laboratory solvents. No data were located on cancer incidence in workers exposed to chloroform vapors.

4.1.2. Exposure to Chloroform in Drinking Water

There have been no studies of toxicity or cancer incidence in humans chronically exposed to chloroform (alone) via drinking water. However, there have been a number of epidemiological studies on cancer risk in humans exposed to chlorinated drinking water (e.g., Cantor et al., 1985; McGeehin et al., 1993; King and Marrett, 1996; Doyle et al., 1997; Freedman et al., 1997; Cantor et al., 1998; Hildesheim et al., 1998). Chlorinated drinking water typically contains chloroform, along with other trihalomethanes and a wide variety of other disinfection byproducts (U.S. EPA, 1994d). It should be noted that humans exposed to chloroform in drinking water are likely to be exposed both by direct ingestion and by inhalation of chloroform gas released from water into indoor air.

Some of these epidemiological studies have detected a weak association between exposure to chlorinated water and cancer (mainly bladder cancer). Based on the studies of Cantor et al. (1985), McGeehin et al. (1993); King and Marrett (1996); Freedman et al. (1997), and Cantor et al. (1998), EPA calculated that the population-attributable risk (the fraction of a disease that could be eliminated if the exposure of concern were eliminated) for bladder cancer ranged from 2% to 17% (U.S. EPA, 1998g). However, these calculations are based on a number of assumptions, including the assumption that there is a cause-effect relationship between exposure to chlorinated drinking water and increased risk of bladder cancer. This assumption is subject to considerable uncertainty, especially because findings are not consistent within or between studies. Evaluation of these studies by application of standard criteria for establishing causality from epidemiological observations (strength of association, consistency of findings, specificity of association, temporal sequence, dose-response relation, biological plausibility) has led EPA to conclude that the current data are insufficient to establish a causal relationship between exposure to chloroform in drinking water and increased risk of cancer (SAB, 2000; U.S. EPA, 1998c; ATSDR, 1997; IPCS, 2000). Moreover, even if, in the future, the weight of evidence does reach a point where a causal link is established between exposure to chlorinated water and increased risk of bladder or other types of cancer, it could not be concluded from epidemiological studies of this type that chloroform per se is carcinogenic in humans, because chlorinated water contains numerous disinfection byproducts besides chloroform that are potentially carcinogenic (U.S. EPA, 1994d, 1998c).

There have also been a number of epidemiological studies that have investigated the association between human exposure to chloroform and other disinfection byproducts in chlorinated water and the occurrence of adverse reproductive outcomes. Several such studies are summarized below:

Study	Study type	Index of exposure	Associated effects
Kramer et al., 1992	Case-control	TTHM (chloroform)	Intrauterine growth retardation
Bove et al., 1995	Cross-sectional	TTHM	Low birth weight Small for gestational age CNS defects Oral cleft defects Cardiac defects
Gallagher et al., 1998	Retrospective	TTHM	Retarded fetal growth
Waller et al., 1998	Prospective	TTHM (BDCM)	Spontaneous abortion

As seen, statistically significant correlations between exposure to total trihalomethanes and one or more adverse reproductive outcomes have been detected in several different types of epidemiological study design. In one case (Kramer et al., 1992), there was a significant relationship between chloroform levels and decreased intrauterine growth. In another case (Waller et al., 1998), an association was noted between increased risk of spontaneous abortion and bromodichloromethane (but not chloroform) levels. As noted earlier, although epidemiological studies of this type are useful in evaluating whether chlorinated drinking water can increase the risk of adverse reproductive effects in exposed populations, the studies are not adequate to establish a causal link between ingestion of chloroform and the occurrence of adverse reproductive effects in humans, because chlorinated drinking water contains many different potentially toxic disinfection byproducts.

4.2. PRECHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS

A number of studies in animals have investigated the chronic toxicity and carcinogenic potential of chloroform. This includes studies both by oral exposure and by inhalation exposure. Presented below are summaries of the most important of these investigations.

4.2.1. Oral Studies

4.2.1.1. *Eschenbrenner, AB; Miller, E. (1945) Induction of hepatomas in mice by repeated oral administration of chloroform, with observations on sex differences. J Natl Cancer Inst 5:251-255.*

Eschenbrenner and Miller (1945) exposed Strain A mice (five/sex/group) to chloroform at dose levels of 0, 150, 300, 600, 1,200, or 2,400 mg/kg in olive oil by gavage,. The animals were dosed every 4 days over a period of 120 days (a total of 30 doses) and were examined for hepatomas 30 days after the last dose. No males administered doses of at least 600 mg/kg and no females in the high-dose group survived the study. All deaths occurred 24 to 48 hours after the first or second chloroform dose. All surviving females dosed with chloroform at 600 or 1,200 mg/kg developed hepatomas. Liver necrosis was observed in both sexes in the three highest dose groups. Necrosis of hepatoma cells was not observed. The hepatomas did not

appear invasive and no metastasis was found. Males in all treatment groups developed kidney necrosis, whereas kidney necrosis was not apparent in any females. The severity of renal necrosis was dose related.

4.2.1.2. *National Cancer Institute (NCI). (1976) Report on carcinogenesis bioassay of chloroform. Springfield, VA: NTIS PB-264018.*

The carcinogenic potential of chloroform was evaluated by NCI (1976) in Osborne-Mendel rats. Male rats were administered concentrations of 90 or 180 mg chloroform/kg/day in corn oil, via oral gavage, 5 days/week for 78 weeks. Female rats were administered concentrations of 125 or 250 mg/kg/day for 22 weeks, after which the doses were reduced to 90 or 180 mg/kg/day, with the average dose over the course of the study being 100 or 200 mg/kg/day. Three additional groups of animals served as matched, colony, and positive controls. At week 111, all rats were sacrificed.

Survival rates and weight gains were decreased for rats in all chloroform treatment groups. A statistically significant increase (24%) in the incidence of kidney epithelial tumors was observed in male rats (12/50) in the high-dose group when compared with males in the control group (0/98). A statistically significant increase in the incidence of thyroid tumors was also observed in female rats, but this finding was not considered biologically significant (U.S. EPA, 1994d).

NCI (1976) also evaluated the carcinogenic potential of chloroform using B6C3F1 mice. The average dose levels for the study were 138 or 277 and 238 or 477 mg/kg/day for males and females, respectively. All mice were sacrificed at weeks 92 or 93. Three additional groups of animals served as matched (20/sex/group), colony (99 males and 98 females), and positive (100/sex/group) controls.

Comparable survival rates and weight gains were observed between the treated and control groups, except for the high-dose females. The incidence of hepatocellular carcinomas was significantly increased in males and females in both the low- and high-dose groups when compared to controls. Many of the male mice in the low-dose group that did not develop hepatocellular carcinoma had nodular hyperplasia of the liver. The incidence of kidney epithelial tumors was comparable between treatment and control groups.

4.2.1.3. *Roe, FJC; Palmer, AK; Worden, AN; et al. (1979) Safety evaluation of toothpaste containing chloroform: I. Long-term studies in mice. J Environ Pathol Toxicol 2:799-819.*

Roe et al. (1979) reported three experiments in mice to evaluate the potential carcinogenicity of chloroform. In three different studies, 10-week-old mice were administered chloroform by gavage 6 days per week for 80 weeks, followed by a 13- to 24-week observation period. The design of each study is summarized below:

Study	Strain (gender)	N	Doses
I	ICI (male, female)	52/sex	17, 60
II	ICI (male)	52	60
III	C57BL, CBA, CF/1, ICI (male)	52 per strain	60

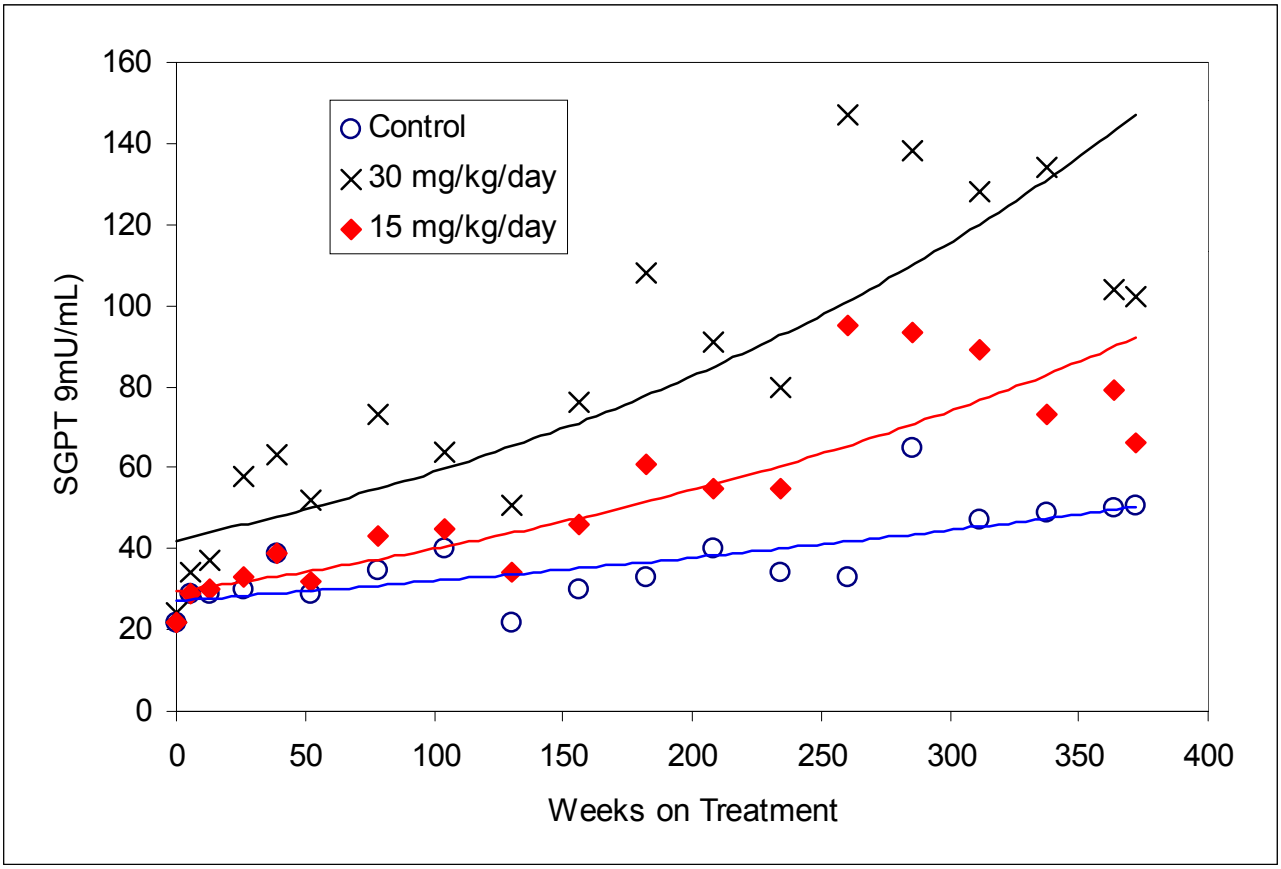
There were no statistically significant differences in survival, body weight, or food consumption between chloroform-treated and control groups in any of the experiments. In experiment I, a slight increase in moderate to severe fatty degeneration of the liver was seen in ICI mice given 60 mg, but not 17 mg, chloroform/kg/day. Kidney tumors were statistically higher in high-dose male mice than in controls, while all other tumor incidences were comparable to control. In experiment II, a decrease in liver and kidney weights was observed in chloroform-treated male mice, and the incidence of kidney tumors was increased. In experiment III, treatment with chloroform was associated with increased incidence of moderate to severe kidney lesions in CBA and CF/1 mice. No increases in liver or kidney tumors were observed except in ICI male mice.

4.2.1.4. *Palmer, AK; Street, AE; Roe, FJC; et al. (1979) Safety evaluation of toothpaste containing chloroform: II. Long-term studies in rats. J Environ Pathol Toxicol 2:821-833.*

Sprague-Dawley rats (50/sex/group) were administered concentrations of 0 or 60 mg chloroform/kg/day in toothpaste by gavage, 6 days/week for 80 weeks. No significant differences in mortality were observed between treated and control animals. A marginal decrease in body weight gain (about 10%) was observed in both treated males and females when compared to controls. A statistically significant decrease in relative liver weight was observed in treated females. Histologic examination of the liver revealed only minor changes, with no severe fatty infiltration, fibrosis, or marked bile duct abnormalities reported. The incidence of moderate to severe glomerulonephritis was reported to be slightly increased in treated males.

4.2.1.5. *Heywood, R; Sortwell, RJ; Noel, PRB; et al. (1979) Safety evaluation of toothpaste containing chloroform: III. Long-term study in beagle dogs. J Environ Pathol Toxicol 2:835-851.*

Heywood et al. (1979) exposed groups of eight male and eight female beagle dogs to doses of 15 or 30 mg chloroform/kg/day. The chemical was given orally in a toothpaste base in gelatin capsules, 6 days/week for 7.5 years. This was followed by a 20- to 24-week recovery period. A group of 16 male and 16 female dogs received toothpaste base without chloroform and served as the vehicle control group. Eight dogs of each sex served as an untreated group and a final group of 16 dogs (8/sex) received an alternative nonchloroform toothpaste. Four male dogs (one each from the low- and high-dose chloroform groups, the vehicle control group, and the untreated control group) and seven female dogs (four from the vehicle control group and three from the untreated control group) died during the study. Results for serum glutamate pyruvate transaminase (SGPT, now known as alanine aminotransferase or ALT) levels are shown in Figure 2. Although there is substantial variability in individual measurements, SGPT levels



Data are from Heywood et al., 1979. SGPT = serum glutamate pyruvate transaminase.

Figure 2. SGPT levels in dogs exposed to chloroform for 7 years.

tended to be about 30%–50% higher in the low-dose group (15 mg/kg/day) than in control animals. These increases were statistically significant for weeks 130-364. For the high-dose group (30 mg/kg/day), the typical increase in SGPT was about twofold, and the differences were statistically significant for the entire exposure duration (weeks 6–372). After 14 weeks of recovery, SGPT levels remained significantly increased in the high-dose group but not in the low-dose group, when compared with the controls. After 19 weeks of recovery, SGPT levels were not significantly increased in either treated group when compared with the controls. The authors concluded that the increases in SGPT levels were likely the result of minimal liver damage. Serum alkaline phosphatase (SAP) and SGPT levels were also moderately increased (not statistically significant) in the treated dogs at the end of the treatment period when compared with the controls. Microscopic examinations were conducted on the major organs. The most prominent microscopic effect observed in the liver was the presence of "fatty cysts," which were described as aggregations of vacuolated histiocytes. The fatty cysts were observed in the control and treated dogs, but were larger and more numerous (i.e., higher incidence of cysts rated as

"moderate or marked," as opposed to "occasional or minimal") in the treated dogs at both doses than in the control dogs. The prevalence of moderated or marked fatty cysts was 1/27 in control animals, 9/15 in low-dose animals, and 13/15 in high-dose animals. Nodules of altered hepatocytes were observed in both treated and control animals, and therefore were not considered related to treatment. No other treatment-related nonneoplastic or neoplastic lesions were reported for the liver, gall bladder, cardiovascular system, reproductive system, or urinary system. A NOAEL was not identified in this study. However, a LOAEL of 15 mg/kg/day was identified, based on elevated SGPT levels and increased incidence and severity of fatty cysts (U.S. EPA, 1998c).

4.2.1.6. *Jorgenson, TA; Rushbrook, CJ. (1980) Effects of chloroform in the drinking water of rats and mice: ninety-day subacute toxicity study. United States Environmental Protection Agency Publication No. EPA-600/1-80-030.*

Seven groups of 6-week-old female B6C3F1 mice (30 mice/group) were given water containing either 0, 200, 400, 600, 900, 1,800, or 2,700 ppm chloroform for 30–90 days. Calculated dose levels were 0, 32, 64, 97, 145, 290, or 436 mg/kg/day based on reported water intakes. At week 1, a significant decrease in body weight was observed in the 900, 1,800, and 2,700 ppm chloroform treatment groups; however, all body weights of the treated animals were comparable to controls after week 1. On days 30, 60, and 90, ten animals from each treatment group were sacrificed for gross and microscopic pathologic examination, as well as for measurement of organ fat:organ weight ratios. A 160%–250% increase in liver fat was observed in the high-dose group. Histological examination of the liver revealed mild centrilobular fatty changes in the 1,800 and 2,700 ppm groups. On day 30, reversible fatty changes in the liver were observed at doses as low as 400 ppm chloroform. Treatment-related atrophy of the spleen was observed at the high dose. Based on the observation of mild effects of chloroform exposure via the drinking water on liver and other tissues, the LOAEL in this study was 290 mg/kg/day, while the NOAEL was 145 mg/kg/day (U.S. EPA 1994d).

4.2.1.7. *Jorgenson, TA; Rushbrook, CJ; Jones, DCL. (1982) Dose-response study of chloroform carcinogenesis in the mouse and rat: status report. Environ Health Perspect 46:141-149.*

This study was an interim report of a 2-year bioassay conducted by Jorgenson et al. (1985) (see below). Male Osborne-Mendel rats and female B6C3F1 mice were exposed to chloroform in drinking water (0, 200, 400, 900, or 1,800 mg/L) for 1-6 months. The time-weighted average doses, based on measured water intake and body weights, were 0, 19, 38, 81, or 160 mg/kg in rats and 0, 34, 65, 130, or 263 mg/kg in mice. An additional group of matched controls received the same water volume as the high-dose groups.

In male rats, some changes were observed in body weight and in some hematological and serum biochemical parameters, but the authors judged these changes to be a secondary effect of reduced water intake. Gross and microscopic pathology findings in the rats generally were slight or mild in severity, were not dose related, and either appeared adaptive (occurred in rats sacrificed after 30 or 60 days, but not in those sacrificed after 90 days) or were sporadic (by

nature and/or incidence) and not considered treatment-related. This study identifies a NOAEL of 160 mg/kg/day in the male rat.

In mice, mortality within the first 3 weeks was significantly increased in the two highest dose groups (130 and 263 mg/kg/day), but was comparable to controls after that time. Early mortality and behavioral effects (e.g., lassitude, lack of vigor) were apparently related to reduced water consumption. A significant increase in liver fat in mice was noted at doses of 65 mg/kg/day and higher at 3 months, and at doses of 130 and 263 mg/kg/day at 6 months. This study identifies a NOAEL of 34 mg/kg/day and a LOAEL of 65-130 mg/kg/day in mice, based on increased liver fat.

4.2.1.8. *Jorgenson, TA; Meierhenry, EJ; Rushbrook, CJ; et al. (1985) Carcinogenicity of chloroform in drinking water to male Osborne-Mendel rats and female B6C3F1 mice. Fundam Appl Toxicol 5:760-769.*

Jorgenson et al. (1985) exposed male Osborne-Mendel rats and female B6C3F1 mice to chloroform in drinking water (0, 200, 400, 900, or 1,800 mg/L) for 104 weeks. Time-weighted average doses, based on measured water intake and body weights, were 0, 19, 38, 81, or 160 mg/kg/day for rats and 0, 34, 65, 130, or 263 mg/kg/day for mice. An additional group of animals that served as controls was limited to the same water intake as the high-dose groups. The number of animals in the dose groups (from low to high) was 330, 150, 50, and 50 for rats and 430, 150, 50, and 50 for mice.

In male rats, survival at 104 weeks was greater in exposed groups than in controls. In female mice, survival was similar to controls following an initial decline in survival of mice that refused to drink for the first week of the study.

A statistically significant dose-related increase in the incidence of kidney tumors (tubular cell adenomas and adenocarcinomas) was observed in male rats in the high-dose group (160 mg/kg). A statistically significant increase in the incidence of lymphomas and leukemias and a statistically significant decrease in the incidence of adrenal cortical adenomas, adrenal pheochromocytomas, and thyroid c-cell adenomas was observed in male rats in the high-dose group when compared with controls. However, study authors suggested that the incidence of renal tumors was the only endpoint that was biologically significant with respect to chloroform treatment (U.S. EPA, 1994d).

Chloroform in the drinking water did not increase the incidence of hepatocellular carcinomas in female B6C3F1 mice. The combined incidence of hepatocellular adenomas and carcinomas was 2% in the high-dose group compared with 6% in the control groups. The authors speculated that the differences observed between this study and the NCI (1976) bioassay may be related to differences in the mode of administration (in drinking water versus in corn oil by gavage).

In reports from the original study (Jorgenson et al., 1982, 1985), histological findings indicative of renal cytotoxicity were not reported. Recently, histological slides of rat kidney from this study have been re-examined to assess whether evidence of renal cytotoxicity could be

detected (ILSI, 1997; Hard and Wolf, 1999; Hard et al., 2000). Based on this reexamination, it was found that animals exposed to average doses of 81 or 160 mg/kg/day of chloroform displayed low-grade renal tubular injury with regeneration, mainly in the mid to deep cortex. The changes included faint basophilia, cytoplasmic vacuolation, and simple hyperplasia in proximal convoluted tubules. In some animals, single-cell necrosis, mitotic figures, and karyomegaly were also observed. Hyperplasia was visualized as an increased number of nuclei crowded together in tubule cross-sections. These changes were observable in the 160 mg/kg/day dose group at 12, 18, and 24 months, and in the 81 mg/kg/day dose group at 18 and 24 months. Cytotoxic changes were not seen in either of the lower dose groups (19 or 38 mg/kg/day). Based on histological evidence of renal cytotoxicity in rats, this study identifies a LOAEL of 81 mg/kg/day.

4.2.1.9. *Bull, RJ; Brown, JM; Meierhenry, EA; et al. (1986) Enhancement of the hepatotoxicity of chloroform in B6C3F1 mice by corn oil: implications for chloroform carcinogenesis. Environ Health Perspect 69:49-58*.

The effect of the vehicle on the hepatotoxicity of chloroform was evaluated using male and female B6C3F1 mice. Doses of 0, 60, 130, or 270 mg/kg/day in corn oil or in 2% emulphor were administered via gavage for 90 days. Based on measurements of serum enzyme levels, serum and tissue triglyceride levels, and histological examination of the livers, the authors concluded that hepatotoxic effects were enhanced by the administration of chloroform via corn oil versus chloroform administered in an aqueous suspension. The authors suggested that the cause may be absorption kinetics or interaction between chloroform and the corn oil vehicle (U.S. EPA, 1994d). A LOAEL of 270 mg/kg/day was identified for chloroform when administered in corn oil, but 270 mg/kg/day was considered a NOAEL for chloroform when administered in aqueous vehicle (U.S. EPA, 1994d).

4.2.1.10. *Tumasonis, CF; McMartin, DN; Bush, B. (1987) Toxicity of chloroform and bromodichloromethane when administered over a lifetime in rats. J Environ Pathol Toxicol Oncol 7:55-64*.

Male and female Wistar rats were administered chloroform in drinking water at concentrations of 0 or 2,900 mg/L for 72 weeks. Concentrations of chloroform were then reduced to 1,450 mg/L for an additional 113 weeks until all animals had died (approximately 185 weeks). The average dose for males and females was approximately 200 or 150 mg/kg/day, respectively (U.S. EPA, 1994d). Exposed animals had a decrease in body-weight gain compared to controls. Treated females (but not males) showed a statistically significant increase in the incidence of hepatic neoplastic nodules, and both males and females had a statistically significant increase in the incidence of hepatic adenofibrosis. It is unclear if the nodules and adenofibroses were considered to be tumors (U.S. EPA, 1994d).

4.2.1.11. *Voronin, VM; Litvirov, NN; Kazachkov, VI. (1987) Carcinogenicity of chloroform in the mouse. Vopr Onkol 33(8):81-85.*

The potential carcinogenicity of chloroform was evaluated in mice following oral administration via oil or water. When administered in oil, 250 mg chloroform/kg/day produced

an increased incidence in tumors (tissue not specified), whereas there were no increases in the incidence of tumors observed in mice treated with 15 mg/kg/day. No increases in tumor incidence were observed in mice treated with up to 42 mg/kg/day via drinking water (U.S. EPA, 1994d).

4.2.1.12. *DeAngelo, A. (1995) Evaluation of the ability of chloroform administered in the drinking water to enhance renal carcinogenesis in male F344 rats (letter summary from A. DeAngelo to N. Chiu, October 1995).*

DeAngelo (1995) exposed male F-344 rats to chloroform in drinking water for 100 weeks. Exposure levels were 0, 900, or 1,800 ppm. Assuming ingestion of about 0.05 L/day of water per kg body weight, this corresponds to doses of approximately 45 and 90 mg/kg/day. Exposure began when the animals were 8–10 weeks of age. Interim sacrifices of groups of 6 animals were performed at 26, 52, and 78 weeks, and the final sacrifice at 100 weeks included 50 animals per group. At each time point, liver and kidney were examined for gross and microscopic lesions.

In the liver, there were borderline significant (p = 0.05-0.10) increases in the prevalence of hepatocellular proliferative lesions at 100 weeks. In addition, there was a statistically significant increase ($p < 0.05$) in the multiplicity of adenomas and carcinomas in the group exposed to 1,800 ppm, and a significant dose trend ($p < 0.05$) for hyperplastic nodules, neoplasia, and total proliferative lesions.

Chloroform conc. in water (ppm)	Hepatocellular proliferative lesions	
	Prevalence	Multiplicity
0	5.6%	0.06
900	2.3%	0.02
1,800	20.5%	0.28

With the exception of midzonal vacuolization (probably due to fat accumulation), there were no hepatic histopathological lesions observed at any of the sacrifice periods other than those normally associated with aging rats. In kidney, a wide variety of chronic nephropathies were observed in both control and exposed animals. The incidence of these nephropathies was not considered to be different than spontaneous background rates. No renal neoplasms were observed in any of the chloroform-exposed groups.

4.2.2. Inhalation Studies

4.2.2.1. *Mery, S; Larson, JL; Butterworth, BE; et al. (1994). Nasal toxicity of chloroform in male F-344 rats and female B6C3F1 mice following a 1-week inhalation exposure. Toxicol Appl Pharmacol 125:214-227.*

Mery et al. (1994) exposed rats and mice to chloroform for 6 hours/day for 7 consecutive days. Exposure concentrations ranged from 1 to 300 ppm. Examination of the nasal passages

revealed that chloroform caused a complex set of responses in the ethmoid turbinates, predominantly in rats. These lesions were most severe peripherally and generally spared the tissue adjacent to the medial airways. The changes were characterized by atrophy of Bowman's glands, new bone formation, and increased labeling index in periosteal cells. The only change noted in the mouse was increased cell proliferation without osseous hyperplasia. The NOAEL values for these responses ranged from 3-100 ppm, with histological and induced cell proliferation being the most sensitive indices of effect.

4.2.2.2. ***Larson, JL; Templin, MV; Wolf, DC; et al. (1996) A 90-day chloroform inhalation study in female and male B6C3F1 mice: implications for cancer risk assessment. Fundam Appl Toxicol 30:118-137.***

Templin, MV; Larson, JL; Butterworth, BE; et al. (1996a) A 90-day chloroform inhalation study in F-344 rats: profile of toxicity and relevance to cancer studies. Fundam Appl Toxicol 32:109-125.

Templin, MV; Constan, AA; Wolf, DC; et al. (1998) Patterns of chloroform-induced regenerative cell proliferation in BDF1 mice correlate with organ specificity and dose-response of tumor formation. Carcinogenesis 19:187-193.

Larson et al. (1996) and Templin et al. (1996a, 1998) performed a series of prechronic studies on the toxicity of inhaled chloroform in B6C3F1 mice and F344 rats. Animals were exposed to concentrations of chloroform ranging from 2-300 ppm (10-1460 mg/m^3) for 6 hours per day, either 5 or 7 days per week, for up to 13 weeks (90 days). All animals were examined for histological lesions of liver, kidney, and nasal epithelium. Some animals were administered bromodeoxyuridine (BrdU) via osmotic pump prior to sacrifice in order to measure the labeling index (LI).

The results of these studies are summarized in Table 2. Exposure to chloroform caused histopathological lesions in liver, kidney, and nasal epithelium of both rats and mice. Lesions in liver were characterized by scattered vacuolated hepatocytes and necrotic foci, sometimes with inflammation, mainly in the centrilobular and midzonal regions. Renal lesions occurred primarily in the epithelial cells of the proximal convoluted tubules in the cortex. Changes included vacuolation, a basophilic appearance, tubule cell necrosis, and enlarged cell nuclei. Nasal lesions were characterized as atrophy of olfactory epithelium, mainly in the ethmoid portion of the nasal passage. In most cases, histological effects in liver and kidney were not observed until exposure levels were about 30 ppm or higher. However, atrophy of the nasal epithelium was observed in rats at the lowest exposure level tested (2 ppm). Histological changes were generally accompanied by statistically significant increases in Labeling Index, although not always at exactly the same exposure level. These increases in Labeling Index are interpreted as evidence that the cytotoxic responses in these tissues triggers a regenerative hyperplasia. Increased cell proliferation was not found in either sex of rats exposed to chloroform for 6 weeks and held (without exposure) until week 13, suggesting that cell proliferation is dependent on the presence of chloroform and represents a regenerative response to cytotoxicity.

Table 2. Summary of chloroform-induced cytotoxicity and cell proliferation via inhalation

Reference	Species	Sex	Exposure duration (days)	Liver				Kidney				Nasal epithelium			
				Histopath.		LI		Histopath.		LI (Cortex)		Histopath.		LI	
				NOAEL, ppm	LOAEL, ppm	NOAEL, ppm	LOAEL, ppm	NOAEL, ppm	LOAEL, ppm	NOAEL, ppm	LOAEL, ppm	NOAEL, ppm	LOAEL, ppm	NOAEL, ppm	LOAEL, ppm
Larson et al., 1996	Mouse B6C3F1	Male	90[a]	10	30	30	90	10	30	10	30	90	--	90	--
			90[b]	10	90	10	90	10	90	--	10	90	--	90	--
		Female	90[a]	10	30	30	90	90	--	90	--	90	--	90	--
			90[b]	10	90	10	90	90	--	90	--	90	--	90	--
Templin et al., 1996a	Rat F344	Male	90[a]	30	90	90	300	30	90	10	30	--	2	2	10
			90[b]	30	90	90	300	90	300	30	90	--	30	--	30
		Female	90[a]	30	90	90	300	90	300	10	30	--	2	2	10
			90[b]	90	300	90	300	90	300	30	90	--	30	--	30
Templin et al., 1998	Mouse BDF1	Male	90[b]	5	30	30	90	5	30	5	30	NA	NA	NA	NA
		Female	90[b]	30	90	30	90	90	--	90	--	NA	NA	NA	NA

[a]Exposure was 7 days/week.
[b]Exposure was 5 days/week.
NOAEL = no-observed-adverse-effect level.
LOAEL = lowest-observed-adverse-effect level.
LI = labeling index.

4.2.2.3. *Nagano, K; Nishizawa, T; Yamamoto, S; et al. (1998) Inhalation carcinogenesis studies of six halogenated hydrocarbons in rats and mice. In: Advances in the prevention of occupational respiratory diseases. Chiyotani, K; Hosoda, Y; Aizawa, Y; eds. Elsevier Science B.V.*

Nagano et al. (1998) evaluated the chronic hepatotoxicity of chloroform in F344 rats and BDF1 mice. This study has also been summarized in abstract form by Yamamoto et al. (1994). Groups of male and female rats and mice were exposed to target chloroform vapor concentrations of 0, 10, 30, or 90 ppm or 0, 5, 30, or 90 ppm, respectively, 6 hours/day, 5 days/week for 104 weeks. To avoid lethality in the high-dose groups, mice in the 30-ppm and 90-ppm groups were exposed to chloroform concentrations of 5 and 10 ppm for 2 weeks each and then 30 ppm for 100 weeks or 5, 10, and 30 ppm for 2 weeks each and then 90 ppm for 98 weeks, respectively. The time-weighted average for the 30-ppm group was 29.1 ppm and for the 90-ppm group 85.7 ppm.

The authors reported that both male and female rats and mice showed necrosis and metaplasia of the olfactory epithelium and goblet cell hyperplasia of the respiratory epithelium. Ossification was observed in the nasal turbinate and in the nasal septum of rats and mice, respectively, at the lowest exposure levels. Statistically significant increases in the incidence of overall renal cell adenoma and renal cell carcinoma were observed in male mice in the 30 (7/50) and 90 (12/48) ppm groups when compared with controls (0/50). The overall incidence rates of renal cell carcinoma were statistically significantly increased in males in the 90-ppm group (11/48) when compared with controls (0/50). There were no statistically significant changes in tumor incidence for female mice or for male or female rats in any exposure group.

Templin et al. (1998) duplicated the exposure regimen in mice (including the acclimatization period) in order to study whether the treatment caused cytotoxicity and regenerative hyperplasia. These authors observed cytotoxicity and hyperplasia in the kidneys of male mice exposed to 30 or 90 ppm throughout a 90-day exposure period. No renal lesions or hyperplasia were observed in female mice. These observations are consistent with the hypothesis that cytotoxicity and regenerative hyperplasia are key events in the neoplastic response to chloroform.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES

4.3.1. Oral Studies

4.3.1.1. *Thompson, DJ; Warner, SD; Robinson, VB. (1974) Teratology studies on orally administered chloroform in the rat and rabbit. Toxicol Appl Pharmacol 29: 348-357.*

A preliminary study was conducted to evaluate embryonic and fetal development of Sprague-Dawley rats administered chloroform in corn oil at doses of 79, 126, 300, 316, or 516 mg chloroform/kg/day via oral gavage on days 6–15 of gestation. Alopecia, rough hair, and eczema were observed in the dams in all dose groups. Significantly decreased food consumption and body weights were noted in dams administered 126 mg/kg/day or greater. Fetotoxicity, acute

toxic nephrosis, hepatitis, and maternal death occurred in animals administered 316 mg chloroform/kg/day and higher.

In the main study, groups of pregnant rats (25/group) were administered 0, 20, 50, or 126 mg chloroform/kg/day via intubation on days 6–15 of gestation. Caesarean section was performed 1 or 2 days prior to expected parturition and fetuses were removed and examined. Maternal toxicity, including decreased weight gain and mild fatty changes in the liver, occurred in dams administered 50 or 126 mg chloroform. A statistically significant increase in the frequency of bilateral extra lumbar ribs and a statistically significant decrease in fetal weight were observed in fetuses from the 126 mg/kg/day dose groups when compared with controls. For the dams, a NOAEL of 20 mg/kg/day and a LOAEL of 50 mg/kg/day were identified in this study. For the fetuses, a NOAEL for this study was 50 mg/kg/day and a LOAEL was 126 mg/kg/day.

Pregnant Dutch-belted rabbits were administered 0, 25, 63, 100, 159, 251, or 398 mg chloroform/kg/day in corn oil on days 6–18 of gestation in a preliminary range-finding study. Results showed decreased maternal survival (60%–100%) in dams administered 100 mg/kg/day or greater. Anorexia, weight loss, diarrhea, abortion, and one maternal death were observed in females administered 63 mg/kg/day. Dams administered 25 mg/kg/day showed signs of mild diarrhea and intermittent anorexia (U.S. EPA, 1994d).

A main study was conducted in which 0, 20, 35, or 50 mg chloroform/kg/day via oral intubation was administered to pregnant rabbits (15/group) on days 6–18 of gestation. Decreased weight gain was reported in dams in the high-dose group. Hepatotoxicity was the cause of four maternal deaths in the high-dose group. No microscopic treatment-related effects were reported in the liver, kidney, or breast of the high-dose dams. A statistically significant decrease in body weight was observed in fetuses from the 20 and 50 mg/kg/day groups when compared with controls. Fetuses from the 20 and 35 mg/kg/day groups had a statistically significant increase in the frequency of incompletely ossified skull bones when compared with controls. However, this effect was not statistically significantly increased when the litter was used as the statistical unit of comparison and in the absence of a dose-response (this effect was not observed in the high-dose group). These findings were not considered evidence of teratogenicity or fetotoxicity by the study authors. Therefore, a NOAEL of 35 mg/kg/day and a LOAEL of 50 mg/kg/day were identified for maternal effects based on the fact that maternal toxicity was observed at doses lower than the doses of chloroform that induced fetotoxicity (U.S. EPA, 1994d).

4.3.1.2. *NTP. (1988) Chloroform reproduction and fertility assessment in CD-1 mice when administered by gavage. Report by Environmental Health Research and Testing, Inc., Lexington, KY, to National Toxicology Program, NTP-89-018. NTIS PB89-148639.*

The reproduction and fertility of CD-1 (ICR) BR outbred albino mice (20/sex/group) administered chloroform in corn oil via gavage at concentrations of 6.6, 16, or 41 mg/kg/day, 7 days/week for 18 weeks was investigated. An additional group of animals (40/sex/group) served as controls. The basis of dose selection was the death of one male animal administered 100 mg/kg/day for 13 days in a range-finding study. Additionally, F_1 mice (20/sex/group) from the

control and high-dose groups were administered the same concentrations of chloroform as their parents from postnatal day 22 until they were sacrificed after the birth of the F_2 generation. Mating of the F_1 generation occurred at 64–84 days of age.

No significant differences in reproductive parameters, such as fertility index, number of litters per pair, litter size, proportion of live pups, proportion of male pups, or pup weight at birth, occurred between treated and control groups. The F_1 generation also had no adverse effects on fertility or reproduction. However, all females in the F_1 generation exposed to 41 mg/kg/day showed increased liver weight and liver lesions characterized by degeneration of centrilobular hepatocytes. Treated males of the F_1 generation had statistically significantly increased epididymal weights, when compared to controls. Sperm motility, sperm density, and percent abnormal sperm were not altered by chloroform treatment in the F_1 generation. However, vacuolar degeneration of ductal epithelium in the cauda epididymis was observed in 8/20 treated and 3/20 control F_1 males. The F_2 generation was not examined microscopically. Study authors concluded that mild to moderate liver histopathology was observed at 41 mg chloroform/kg/day in F_1 females but not males, and that minimal epididymal histopathology was observed in F_1 males. A NOAEL could not be defined in this study because histopathology was not performed on animals in the low- and mid-dose levels.

4.3.1.3. *Ruddick, JA; Villeneuve, DC; Chu, I. (1983) A teratological assessment of four trihalomethanes in the rat. J Environ Sci Health 18(3):333-349*.

A study was conducted to determine the potential developmental toxicity of chloroform following administration via oral gavage in rats. On gestational days 6 through 15, pregnant dams (8 to 14 animals/dose group) were administered 0, 100, 200, or 400 mg chloroform/kg in corn oil. On day 22 of gestation, dams were anesthetized with ether and their viscera, including the uteri, were examined. The fetuses were removed, weighed, and examined for viability and external malformations. Histological examination was performed on two fetuses from each dam. Maternal endpoints evaluated included hematology (hemoglobin concentration, erythrocyte and leucocyte counts, hematocrit, mean corpuscular hemoglobin concentration, and mean corpuscular hemoglobin), clinical chemistry (alkaline phosphatase, sodium, total bilirubin, cholesterol, glucose, potassium, inorganic phosphorus, calcium, uric acid, LDH, GOT, and total protein), and gross examination of the organs.

A significant decrease in weight gain, hemoglobin levels, and hematocrit levels as well as enlargement of the liver in dams occurred at all dose levels. A significant increase in serum inorganic phosphorus, cholesterol levels, and kidney weights and a decrease in RBC count were observed in dams in the high-dose group. Also in the high-dose group, a statistically significant (19%) decrease in fetal body weight was observed when compared with the controls. There were no fetal malformations upon gross examination; however, a dose-dependent increase in the incidence of sternebra aberrations was observed in the 200 and 400 mg chloroform/kg/day exposure groups. Deviations were also observed at the high dose. However, statistical analyses were not performed on the observed variations.

4.3.2. Inhalation Studies

4.3.2.1. *Baeder, C; Hoffman, T. (1988) Initial submission: inhalation embryotoxicity study of chloroform in Wistar rats (final report) with attachment and cover letter dated 02/21/92. Pharma Res Toxicol Pathol. Conducted for Occidental Chem Corp. U.S. EPA/OTS Public Files, Document Number: 88-920001208.*

The potential developmental toxicity of chloroform vapor was evaluated following inhalation exposure in rats. On gestational days 7 to 16, groups of 20 pregnant Wistar rats were exposed to 0, 30, 100, or 300 ppm (0, 146, 488, 1,464 mg/m^3) chloroform via inhalation for 7 hours/day. On gestational day 21, dams were sacrificed and fetuses were removed by Caesarian section, weighed, sexed, and measured for crown-rump length. Half of the fetuses were examined for skeletal anomalies, while the other half were examined for organ anomalies. Maternal endpoints evaluated included food consumption, body weight, clinical signs of toxicity, selected organ weights (heart, liver, kidneys, and spleen), and reproductive viability (number of live and dead fetuses, number of corpora lutea, embryonic resorption sites, and placentas).

A dose-related decrease in maternal food consumption with increasing chloroform concentrations occurred throughout the gestational period. On gestational days 14, 17, and 21, maternal body weight and body weight gain values (18%, 24%, and 29% at 30, 100, and 300 ppm, respectively) were also decreased in a concentration-related manner when compared to controls. A significant decrease (6%) in mean fetal weights was observed for the high-concentration group. At all exposure concentrations, an increase in the number of dead fetuses (there were no live fetuses in 2 dams at 30 ppm, 3 dams at 100 ppm, and 8 dams at 300 ppm) and a significant decrease in fetal crown-rump length was observed. Fetal skeletal development for all treatment groups was comparable to controls. Based on maternal toxicity and fetal lethality, the study authors identified a LOAEL of 30 ppm (146 mg/m^3). This corresponds to a time-weighted average concentration of 43 mg/m^3. A NOAEL was not identified for this study.

4.3.2.2. *Baeder, C; Hoffman, T. (1991) Initial submission—chloroform: supplementary inhalation embryotoxicity study in Wistar rats (final report) with attachments and cover letter dated 12/24/91. NTIS/OTS0535017. EPA/OTS Doc#8-920000566. Study title: Chloroform: supplementary inhalation embryotoxicity study in Wistar rats. September 12, 1991. Performed by Hoechst Aktiengesellschaff, Germany, Sponsored by Hoechst AG and Dow Europe SA. Report No. 91.0902.*

Baeder and Hoffmann (1991) exposed groups of 20 pregnant Wistar rats to 0, 3, 10, or 30 ppm chloroform via inhalation for 7 hours/day on gestational days 7 to 16. The actual delivered concentrations of chloroform were 0, 3.1, 10.7, or 30.2 ppm (0, 15, 52.2, or 147 mg/m^3). On gestational day 21, dams were sacrificed and fetuses were removed by Caesarian section, weighed, sexed, and measured for crown-rump length. Half of the fetuses were examined for skeletal anomalies while the other half were examined for internal anomalies. Maternal endpoints examined included food consumption, body weight, clinical signs of toxicity, selected organ weights (heart, liver, kidneys, and spleen), and reproductive viability (number of live and dead fetuses, resorptions, corpora lutea, and placentas). Maternal food consumption was significantly decreased in all exposure groups, and maternal body weight was significantly

decreased in the 10-and 30-ppm treated groups. A concentration-related decrease in overall body weight gains for dams for all exposure groups was reported. At 30-ppm, significant increases in maternal kidney weights and significant decreases in fetal body weights and crown-rump lengths were observed. One dam exposed to 30-ppm chloroform via inhalation exhibited only empty implantation sites (i.e., no fetuses were present). A statistically significant increase in the incidence of fetuses with body weights <3 grams and in the incidence of fetuses with slight or no ossification of individual skull bones was observed in the 30-ppm exposed group when compared with controls. The incidence of fetuses with body weights <3 grams was increased in a dose-related fashion (3.2%, 14.2%, 24%, and 26.9% at 0, 3, 10, and 30-ppm, respectively); this trend did not appear to be due to variations in litter size. However, when the litter was used as the statistical unit of comparison, only litters from the high-concentration group had a significant number of fetuses weighing 3 grams or less. A significant increase in the incidence of fetuses with ossification of less than two caudal vertebral centers was observed at all concentrations. A dose response was observed for the incidence of litters with this effect; however, the effect was not statistically significant. Finally, all exposure groups exhibited a significant increase in the incidence of both litters and fetuses with nonossified or weakly ossified sternebrae; however, there was no statistically significant concentration-related trend for this effect. Based on decreased body weight gain in dams, and slight retardation in growth of fetuses, a NOAEL of 3 ppm and a LOAEL of 10 ppm were identified. Even though there were increases in low-weight fetuses at the two lowest concentrations, this effect was not considered adverse. Therefore, the NOAEL for developmental effects in this study was 10.7 ppm (52.2 mg/m^3) based on the weight of evidence of the data, including comparison to historical controls and the higher concentration study.

4.3.2.3. *Schwetz, BA; Leong, BJK; Gehring, PJ. (1974) Embryo- and fetotoxicity of inhaled chloroform in rats. Toxicol Appl Pharmacol 28:442-451.*

Four groups of 68, 22, 23, and 3 pregnant Sprague-Dawley rats were exposed to either 0, 30, 100, or 300 ppm chloroform, respectively, via inhalation for 7 hours/day from gestational days 6 to 15. Because in an earlier experiment marked anorexia was observed in dams exposed to 300 ppm chloroform, an additional control group (starved) that was allowed only 3.7 grams of food per day was also used. Actual delivered concentrations of chloroform were 0, 30, 95, or 291 ppm (0, 146, 464, or 1,420 mg/m^3). The low percent pregnancy observed at the high-concentration group was not considered to be treatment-related because of the timing of exposure; however, the use of such a small number of animals in the 300-ppm group decreased the statistical sensitivity of any adverse effects observed in this group. On gestational day 21, dams were sacrificed and fetuses were removed by Caesarian section, weighed, measured, and sexed. Half of the fetuses were examined for skeletal anomalies while the other half were examined for organ anomalies. A concentration-related decrease in body weight gain and food consumption was observed in dams of all exposure groups. A significant increase in relative liver weights in dams exposed to 100- and 300-ppm chloroform was observed at study termination, with a significant decrease in absolute liver weight reported in dams exposed to 300 ppm chloroform. In the high-concentration group, 61% of the implantations were resorbed (statistically significant). This high resorption rate was not observed in the “starved” control group; therefore, weight loss cannot account for the observed effect. Fetal body weights (40%) and fetal crown-rump lengths were significantly decreased at 300 ppm. Fetal crown-rump

lengths were significantly decreased in the 30- and 300-ppm groups by 2% and 15%, respectively. At 100 ppm chloroform, the frequencies of litters with acaudia or imperforate anus were significantly increased when compared with the controls. All exposure groups exhibited an increase in the frequency of litters with delayed ossification. Also, there were statistically significant increases in wavy ribs at 30 ppm and in missing ribs and subcutaneous edema at 100 ppm. Fetal malformations were not observed at the high-dose group; however, there were only three litters at this concentration. The study authors concluded that exposure to 100 and 300 ppm chloroform via inhalation was embryotoxic and fetotoxic, with embryo death a significant effect at 300 ppm.

4.3.2.4. *Murray, FJ; Schwetz, BA; McBride, JG; et al. (1979) Toxicity of inhaled chloroform in pregnant mice and their offspring. Toxicol Appl Pharmacol 50:515-522.*

The potential developmental toxicity of chloroform vapor was investigated following inhalation exposure in mice. Groups of 34 to 40 pregnant CF-1 mice were exposed to either 0 or 100 ppm chloroform (0 or 490 mg/m^3) 7 hours/day on gestational days 1–7, 6–15, or 8–15, and sacrificed on gestational day 18. Chloroform exposure at 100 ppm was teratogenic in mice exposed on gestational days 8–15, and fetotoxic in mice exposed on gestational days 1–7 or 6–15. A significant decrease in the ability of mice to maintain pregnancy was observed in the group exposed on gestational days 1–7 or 6–15, and there was a slight, but not statistically significant, decrease in pregnancies in the group exposed on gestational days 8–15. Fetal weight and length were significantly decreased in the groups exposed on gestational days 1–7 and 8–15, but not 6–15. A significant increase in the incidence of litters with cleft palate and delayed ossification of sternebrae was observed in the gestational day 8–15 exposed group or all exposure groups, respectively. Cleft palate was not observed in the gestational day 1–7 or the 6–15 exposed groups and was mostly in those fetuses with retarded growth. Delayed ossification of skull bones was significantly increased in all exposure groups. A significant increase in the incidence of delayed ossification of the sternebrae was reported in the group exposed on gestational days 1–7 and 8–15, but not days 6–15. No other malformations were significantly increased in any chloroform treatment group. The study authors suggested that the lack of malformations in the gestational day 6–15 exposed group may have been due to the lethality of chloroform on the early embryo. Liver weights and SGPT activity were increased in dams exposed to chloroform.

4.4. OTHER STUDIES

4.4.1. Other Effects

As summarized above, the available data indicate that the characteristic effects of chloroform exposure include cytotoxicity in liver, kidney, and nasal epithelium, with neurological effects following relatively high-dose inhalation exposures. No studies were located that identified toxic effects on other tissues such as the immune system.

4.4.2. Mutagenicity

4.4.2.1. *Overview*

A number of studies have been performed to evaluate the mutagenicity of chloroform. In reviewing and evaluating these studies, it is important to recognize the following potential concerns regarding study design: (1) because chloroform is relatively volatile, test systems not designed to prevent chloroform escape to the air may yield unreliable results; (2) because it is the metabolites of chloroform (e.g., phosgene, dichloromethyl free radical) rather than the parent compound that are most likely to react with DNA, studies in which appropriate P450-based metabolic activation systems are absent are also likely to be unreliable; (3) because of the relatively high reactivity of the metabolites, tests using exogenous activation systems (i.e., the metabolites are formed outside of the test organism) are likely to be less relevant than tests using endogenous activation systems (i.e., metabolites are formed inside the test organism); (4) studies (especially older studies) that used ethanol as a solvent or preservative for chloroform may be confounded by formation of ethyl or diethyl carbonate, which are potent alkylating agents; and (5) tests performed in vitro (e.g., clastogenicity tests) or in vivo under highly toxic doses can produce positive results as a secondary consequence of severe cytotoxicity, resulting from lysosomal or other releases (Brusick, 1986). Also, chloroform-induced cycles of cytotoxicity and cell proliferation in vivo or in vitro could cause the expression of preexisting genetic damage in cells that, under normal conditions, have low mitotic indices. Therefore, one should exercise caution in interpreting the mutagenicity test results.

4.4.2.2. *Evaluation of Available Data*

4.4.2.2.1. ***In Vitro Studies.*** Data on the genotoxic potential of chloroform in subcellular systems are limited, but two investigators reported DNA binding in studies with calf thymus DNA in the presence of exogenous activation (DiRenzo et al., 1982, Colacci et al., 1991). The study by DiRenzo et al. (1982) utilized ethanol as a solvent, suggesting that ethyl carbonate formation might be a problem. In the study by Colacci et al. (1991) addition of SKF-525A inhibited DNA binding, suggesting that binding was mediated by a cytochrome P-450 pathway, as would be expected for chloroform. In interpreting these studies, it is important to remember that cell-free systems may not always be a good model for intact cellular processes.

Gene mutation studies in *Salmonella typhimurium* and *E. coli* (Ames assay), including tests done under conditions designed to reduce evaporation, are mostly negative, with or without activation with microsomes from liver or kidney of rats or mice (Rapson et al., 1980; San Agustin and Lim-Sylianco, 1978; Van Abbe et al., 1982; Uehleke et al., 1977; Gocke et al., 1981; Roland-Arjona et al., 1991; Le Curieux et al., 1995; Kirkland et al., 1981; Simmon et al., 1977). However, four studies have showed positive results in bacteria. Varma et al. (1988) reported that chloroform caused mutagenicity in five strains of *S. typhimurium*, but the response was noted only at the lowest dose tested, and all higher doses were no different from control. This unusual pattern casts some doubt on these results. San Agustin and Lim-Sylianco (1997) reported that chloroform caused DNA damage in *Bacillus subtilis*, and Wecher and Scher (1982) reported that chloroform caused mutations in *Photobacterium phosphoreum*. However, neither study reported the exposure concentrations that caused these effects, so the relevance of these reports is

uncertain. In addition, the studies by Varma et al. (1988) and Wecher and Scher (1982) each used ethanol as a diluent, raising the possibility that the positive effect might be related to ethyl carbonate formation rather than to chloroform. The majority of results reported for *S. typhimurium* and *E. coli* exposed to the vapor phase were also negative (Van Abbe et al., 1982; Pegram et al., 1997; Simmon, 1977; Sasaki et al., 1998). Pegram et al. (1997) reported that chloroform was weakly positive at vapor concentrations greater than 19,200 ppm (about 770 mg/L in the aqueous phase). Employing physiologically based pharmacokinetic models, the authors estimated the oral doses needed to produce the effect would exceed 2,000 mg/kg (approximately twice the LD50).

Tests of genotoxicity are also mainly negative in fungi (Gualandi, 1984; Mehta and von Bortsel, 1981; Kassinova et al., 1981; Jagannath et al., 1981). However, chloroform was shown to induce intrachromosomal recombination in *Saccharomyces cerevisiae* at concentrations of 6,400 mg/L (Callen et al., 1980) or 750 mg/L (Brennan and Schiestl, 1998). In the Brennan and Schiestl study, addition of *N*-acetylcysteine reduced chloroform-induced toxicity and recombination, suggesting a free radical may have been involved. Chromosome malsegregation was also reported in *Aspergillus nidulans* (Crebelli et al., 1988), but only at concentrations above 1,600 mg/L. In all three of these positive studies, doses that caused positive results also caused cell death, indicating that exposures were directly toxic to the test cells.

Studies in intact mammalian cells are mainly negative (Larson et al., 1994a; Perocco and Prodi, 1981; Butterworth et al., 1989; Kirkland et al., 1981; White et al., 1979; Sturrock, 1977), although positive results have been reported in a few systems. Increased sister chromatid exchange was reported in human lymphocytes at a concentration of about 1,200 mg/L without exogenous activation (Morimoto and Koizumi, 1983), and at a lower concentration (12 mg/L) with exogenous activation (Sobti, 1984). In the study by Sobti, the increase was quite small (less than 50%), and there was an increase in the number of cells that did not exclude dye. This suggests that the exposure levels causing the mutagenic effect may have been directly toxic to the cells. In addition, ethanol was used as a dose vehicle. Mitchell et al. (1988) did not detect an increase in mutation in mouse lymphoma cells at an exposure level of 2,100 mg/L in the absence of exogenous activation, but did detect an effect at a concentration of 59 mg/L with exogenous activation.

4.4.2.2.2. ***In vivo studies.*** A number of different endpoints of chloroform genotoxicity have been measured in intact animals exposed to chloroform either orally or by inhalation. In studies of DNA binding in liver and kidney of mice and rats, negative results have been reported at doses of 742 mg/kg, 119 mg/kg, and 48 mg/kg (Diaz Gomez and Castro, 1980; Reitz et al., 1982; Pereira et al., 1982). However, positive results have been reported at doses as low as 2.9 mg/kg (Colacci et al., 1991). In the study by Colacci et al (1991), no significant difference in binding was noted between multiple tissue (liver, kidney, lung, and stomach), and there was no increase in binding with phenobarital pretreatment. This suggests the binding may not have been related to chloroform metabolism.

Studies based on signs of DNA damage or repair have been uniformly negative (Larson et al., 1994a; Potter et al., 1996; Reitz et al., 1982; Mirsalis et al., 1982). However, studies based on various signs of chromosomal abnormalities have been mixed, with some studies reporting

negative findings at doses of 371 mg/kg and 800 mg/kg (Shelby and Witt, 1995; Topham, 1980), while other studies report positive results at doses as low as 1.2 mg/kg (Fujie et al., 1990). However, the positive result at low dose in the study by Fujie et al. (1990) was observed following intraperitoneal exposure, and positive results following oral exposure were not observed until dose levels of 119 mg/kg. Morimoto and Koizumi (1983) observed an increase in the frequency of sister chromatid exchange in bone marrow cells at a dose of 50 mg/kg/day, but at 200 mg/kg/day, all of the mice died. As discussed before, mutagenicity results observed following highly toxic doses may have been confounded by cytotoxic responses and should be viewed as being of uncertain relevance.

Several studies have reported negative findings for the micronucleus test in rats and mice (Gocke et al., 1981; Salamone et al., 1981; Le Curieux, 1995), but several other studies have detected a positive result, mainly at exposure levels of 400-600 mg/kg (San Agustin and Lim-Sylianco, 1982; Robbiano et al., 1998; Sasaki et al., 1998; Shelby and Witt, 1995). This suggests that chloroform may be clastogenic, but it is important to note that these doses are well above the level that causes cytotoxicity in liver and kidney in most oral exposure studies in rodents.

Butterworth et al. (1998) did not detect an increase in mutation frequency in male mice exposed by inhalation at an exposure level of 90 ppm, even though this exposure did cause an increase in tumors in the study by Nagano et al. (1998). Increased incidence of spermhead abnormalities was reported in mice exposed at 400 ppm (Land et al. 1981), but were not observed in mice exposed to 371 mg/kg intraperitoneally (Topham 1980).

In *Drosophila melanogaster* larvae exposed to chloroform vapor, gene mutation (Gocke et al. 1981) and mitotic recombination tests (Vogel and Nivard 1993) were both negative. Grasshopper embryos (*Melanoplus sanguinipes*) did not display mitotic arrest at vapor concentrations of 30,000 ppm, but an effect was seen at 150,000 ppm (Liang et al. 1983). San Agustin and Lim-Syllianco (1981) reported a single positive and negative result for host-mediated mutagenicity in *Salmonella typhimurium*, but exposure levels were not reported in either case.

4.4.2.3. *Reviews by Other Groups*

Data on the mutagenicity of chloroform have recently been reviewed and evaluated by several groups, including the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC), ILSI (1997), and WHO (1998). The findings of these review efforts are summarized in the following paragraphs.

4.4.2.3.1. ***ICPEMC.*** The ICPEMC has developed a comprehensive, quantitative weight-of-evidence approach for assessing genotoxic potential and has used this approach to evaluate more than 100 chemicals with large genetic toxicity databases (Lohman et al., 1992). In this approach, scores are developed for relative DNA reactivity. For a particular chemical, the maximum possible score is 100 and the minimum possible score is –100. The highest actual score obtained using this approach was 49.7 (triazaquone) and the lowest score was –27.7 (ethanol). When this approach was applied to chloroform, the score based on the results of more than 40 studies was

–14.33. Thus, ICPEMC concluded that the weight of evidence indicates that chloroform should be classified as nongenotoxic (Brusick et al., 1992; Lohman et al., 1992).

4.4.2.3.2. ***ILSI.*** ILSI (1997) performed a review of the available data on the mutagenicity of chloroform. The committee noted that phosgene is highly reactive and might be expected to have the capacity to interact directly with DNA, but that phosgene has not been tested in any standard mutagenicity test system. The committee also noted that, because of its high reactivity, phosgene formed in the cytosol following chloroform metabolism would likely react with cellular components prior to reaching the cell nucleus, and concluded that direct effects on DNA would be unlikely. Based on their review of the available data, the ILSI committee (ILSI, 1997) concluded that no subset of observations points unequivocally to a specific genotoxic mode of action associated with chloroform, and that the preponderance of the evidence indicates that chloroform is not strongly mutagenic. Based on this, the committee concluded that chloroform would not be expected to produce rodent tumors via a genotoxic mechanism.

4.4.2.3.3. ***WHO.*** WHO (1998) noted that studies on the mutagenicity of chloroform must be considered in light of the fact that (1) chloroform is volatile, so tests that do not prevent volatilization are unreliable, and (2) most chloroform contains ethanol, which may react with phosgene generated from chloroform metabolism to yield ethyl or diethyl carbamates (potentially causing false positive results). The WHO committee noted that largely negative results have been obtained in *Salmonella typhimurium* and *Escherichia coli* (with and without activation), in gene mutation tests in CHO cells and human lymphocytes, in mouse micronucleus tests, and in tests of unscheduled DNA synthesis both in vitro and in vivo. Given the large number of sensitive assays that have been used to investigate the genotoxicity of chloroform, the committee considered it noteworthy that the positive responses were so few, and that the positive results were randomly distributed among the various assays. Taken together, WHO (1998) concluded that the weight of evidence indicates that neither chloroform nor its metabolites appear to interact directly with DNA or possess genotoxic activity.

4.4.2.4. *Overall Weight-of-Evidence Conclusion on Mutagenicity*

In summary, the results of the mutagenicity assays that have been conducted with chloroform are mixed. By number, the majority of tests are negative, and many of the positive studies have been conducted under high exposure conditions that resulted in severe cytotoxicity. As expressed in SAB (2000):

> *Genotoxicity endpoints have to be interpreted cautiously when used as evidence for potential carcinogenicity. In vitro clastogenicity can be a product of severe cytotoxicity resulting from lysosomal or other releases (Brusick, 1986). This may be important with substances such as chloroform, where there is evidence of cytotoxicity and cell proliferation in target tissues. Also, cycles of cytotoxicity and cell proliferation could cause the expression of preexisting genetic damage in target tissues which, under normal conditions, have low mitotic indices.*

Consequently, the relevance of many of the positive studies is questionable. Therefore, based on the preponderance of negative findings and the uncertain relevance of the positive findings, EPA

concludes that the weight of evidence indicates that even though a role for mutagenicity cannot be excluded with certainty, chloroform is not a strong mutagen and that neither chloroform nor its metabolites readily bind to DNA. Based on these results and the results of studies that evaluated other endpoints of DNA reactivity, it seems likely that chloroform does not produce carcinogenic effects primarily by a specific mutagenic mode of action.

4.4.3. Studies Related to Mode of Action

The precise mode of action by which chloroform produces toxic effects is not yet certain, but it is evident that metabolism of chloroform to toxic metabolites plays a critical role (U.S. EPA, 1994d). Representative studies that provide information on the role of metabolism in chloroform-induced toxicity and on the mechanism of metabolism-induced toxicity are summarized below.

4.4.3.1. *Studies That Demonstrate That Metabolism is Required for Toxicity*

A large number of studies support the conclusion that metabolism of chloroform is required for toxicity. Brown et al. (1974) reported that pretreatment of rats with phenobarbital (a cytochrome P-450 inducer) resulted in increased hepatic toxicity following chloroform exposure. Similarly, Gopinath and Ford (1975) indicated that chloroform hepatotoxicity in rats was increased by phenobarbitone, phenylbutazone, and chlorpromazine, all inducers of microsomal enzymes. Conversely, inhibitors of microsomal enzymes, such as SKF-525A, sodium diethyl-dithiocarbamate, and carbon disulfide, decreased the hepatic toxicity of chloroform. Constan et al. (1999) showed that 1-aminobenztriazole, which is a general cytochrome P450 inhibitor, prevented chloroform-induced toxicity in liver and kidney of mice following inhalation exposure.

ILSI (1997) summarized several studies to correlate the degree of hepatic metabolism with toxicity. Pohl and Krishna (1978) indicate that metabolism is essential for chloroform to induce liver toxicity in rats and mice. Smith and Hook (1983) evaluated the nephrotoxicity of chloroform in kidney slices. At equimolar concentrations, ^{1}H-chloroform, which is readily metabolized, induced nephrotoxicity more readily than ^{2}H-chloroform, which is not as readily metabolized as ^{1}H-chloroform.

Further evidence of the role of metabolism is derived from the finding that variations in toxicity between tissues, genders, and species generally correlate with differences in metabolic rate. For example, male mice are more sensitive to chloroform-induced renal toxicity than female mice, and this difference in toxicity is paralleled in a difference in metabolism in proximal tubular cells (Ilett et al., 1973). Renal cytochrome levels in mice are increased by testosterone (Mohla et al., 1988, Henderson et al., 1989; Hong et al., 1989), and male mice are more sensitive to chloroform-induced renal toxicity than are females. Female mice treated with testosterone have increased renal toxicity along with increased covalent binding of chloroform metabolites (Taylor et al., 1974; Smith et al., 1979; Pohle et al., 1984). Conversely, male mice that were castrated had lower levels of chloroform-derived radioactivity accumulated in the kidneys (Eschenbrenner and Miller, 1945; Culliford and Hewitt, 1957; Taylor et al., 1974; Smith et al., 1984).

4.4.3.2. *Identification of Specific Enzymes Responsible for Metabolism*

Nakajima et al. (1995a,b) evaluated the magnitude and localization of liver tissue injury in mice pretreated with chemicals known to induce specific P450 enzymes. These chemicals were n-hexane, an inducer of CYP2E1, phenobarbital, an inducer of CYP2B1/2, and 2-hexanone, an inducer of both enzymes. Hepatocyte necrosis was associated more with CYP2B1/1 induction whereas ballooning of cells was observed more frequently with CYP2E1 induction. The results of the histologic examinations indicated that liver damage was associated more with CYP2E1 induction, with the damage localized primarily to the centrilobular regions, than with CYP2B1/2 induction, where damage was more generalized. At low doses, chloroform is metabolized more extensively by CYP2E1 and activity of this enzyme correlates with tissue damage (ILSI, 1997).

Most recently, Constan et al. (1999) compared the toxicity of chloroform in three strains of mice: B6C3F1, Sv/129 wild type, and Sv/129 CYP2E1 knockout mice. Exposure to 90 ppm chloroform for 6 hrs/day for 4 days produced clear hepatotoxicity and renal toxicity (histopathology, increased labeling index) in the B6C3F1 mice and the Sv/129 wild type, but not in the Sv/129 CYP2E1 knockout mice. The authors concluded that metabolism of chloroform by CYP2E1 was obligatory for toxicity, at least at the dose tested.

4.4.3.3. *Role of Covalent Binding*

The precise mode of action by which chloroform metabolism leads to cell toxicity is not known with certainty, but covalent binding of phosgene with key cellular molecules is considered to be a likely pathway. Pohl et al. (1980) reported that the level of covalent binding correlated directly with injury to the liver tissue and concluded that phosgene was the metabolite responsible for the covalent binding to liver macromolecules. Brown et al. (1974) reported that pretreatment of rats with phenobarbital (a cytochrome P-450 inducer) resulted in increased formation of covalent adducts and increased hepatic toxicity following chloroform exposure. Studies by Ilett et al. (1973) and Tyson et al. (1983) also show that covalent binding to proteins in rats and mice is more prevalent in areas of necrosis than in areas where tissue damage is not severe. DBA mice have a metabolism rate twice as fast as that of C57BL mice (Pohl et al., 1984) and also much greater covalent binding to renal microsomes (Clemens et al., 1979). The results of *in vitro* studies also indicate that metabolism is necessary for covalent binding to macromolecules (Cresteil et al., 1979).

4.4.3.4. *Role of Glutathione*

Reaction of chloroform metabolites (phosgene) with glutathione is a probable detoxification pathway. Acute chloroform toxicity is associated with glutathione depletion (Brown et al., 1974; Stevens and Anders, 1981), and it has been reported that glutathione levels decrease in a dose-dependent manner prior to microscopic evidence of liver pathology (Brown et al., 1974; Docks and Krishna, 1976). Glutathione depletion was reported in chloroform-exposed mice pretreated with phenobarbital; however, this effect was not observed in mice that received chloroform alone (Brown et al., 1974). The results of in vitro studies indicate that glutathione inhibits covalent binding in liver cells (Cresteil et al., 1979; Sipes et al., 1977; Smith and Hook, 1984). After glutathione depletion, continued chloroform exposures resulted in increased

covalent binding and lipid peroxidation (Brown et al., 1974). Glutathione depletion has been observed primarily following acute high exposures or in phenobarbital-treated rats (ILSI, 1997), and not in animals exposed to lower doses over a longer period of time (Munson et al., 1982).

4.4.3.5. *Role of Dose Vehicle*

The results of some animal studies have suggested that the vehicle used to administer chloroform may affect the toxicity (Bull et al., 1986; Jorgenson et al., 1985; Lilly, 1992; Larson et al., 1994b, 1995a). Lilly (1992) reported that hepatic and renal toxicity was greater in rats that received chloroform in a corn oil vehicle when compared with the toxicity observed in rats that received chloroform in an aqueous vehicle. Similar results were reported by Bull et al. (1986), in that hepatotoxicity was greater in rats that received chloroform via corn oil for 90 days, when compared with rats that received chloroform in water. Larson et al. (1994b, 1995a) indicated that following the administration of chloroform via corn oil gavage, there were dose-related increases in centrilobular necrosis and hepatic cell proliferation in female B6C3F1 mice and male F344 rats. These effects were not observed in rats that received chloroform via drinking water. The authors suggested that these differences may have been due to the delivery of a higher dose to the liver following a single gavage dose, when compared to the delivery of smaller doses over a prolonged period as would be the case with administration via drinking water.

4.4.3.6. *Studies on Initiation-Promotion of Cancer Effects*

ILSI (1997) reviewed studies that have been conducted to evaluate the potential for chloroform to promote tumor formation when administered in initiation-promotion protocols (Pereira et al., 1982; Klaunig et al., 1986; Herren-Freund and Pereira, 1986; Reddy et al., 1992; Oesterle and Deml, 1985). The results of the studies by Klaunig et al. (1986), Herren-Freund and Pereira (1986), and Reddy et al. (1992) indicated that chloroform, when administered in the drinking water, did not promote the development of liver tumors in rats or in two strains of mice, that chloroform in some cases inhibited the development of hepatic lesions, and that chloroform did not act as initiator or cocarcinogen. Pereira et al. (1982) reported that chloroform, when administered as a single dose of 180 mg/kg in tricaprylin, did not demonstrate initiating activity. However, a significant increase in GGT-positive hepatic foci was reported in rats initiated with diethylnitrosamine (DEN) and treated with 180 mg chloroform/kg twice weekly for 2 months. Oesterle and Deml (1985) reported that chloroform had initiating activity, as indicated by increased incidences of GGT-positive and ATPase-deficient lesions in the livers of female rats initiated with DEN and treated with chloroform in olive oil.

4.4.3.7. *Role of Altered Gene Expression in Carcinogenicity*

Several studies have investigated the potential for chloroform to alter gene expression as a carcinogenic mode of action. These studies have been summarized by ILSI (1997) and U.S. EPA (1998c). Fox et al. (1990) evaluated the mutation frequency of the H-ras oncogene in liver tumors in male B6C3F1 mice, which have a high spontaneous incidence of liver tumors (2%–30%). In the spontaneous tumors, mutations activating the H-ras oncogene were present in about 64% of the tumors. However, in mice treated with 200 mg chloroform/kg twice weekly via corn oil gavage for 1 year, only about 21% of the liver tumors had mutations that activated the H-

ras oncogene. Based on these results, the authors concluded that mutations activating the H-ras oncogene may be a mechanism for the formation of spontaneous tumors, but that chloroform-induced liver tumors occurred by a different mechanism.

The expression of various oncogenes in the liver was evaluated by Sprankle et al. (1996). Oncogene expression was evaluated in the livers of B6C3F1 mice that had received 350 mg chloroform/kg and rats that received 180 mg/kg via a single oral corn oil gavage. In the female mouse liver, transient increases in mRNA for the *myc* and *fos* genes were reported; however, mRNA levels for the Ha-ras and *met* genes and for hepatocyte growth factor were similar to the levels observed in the controls. The authors noted similar gene responses were reported for other carcinogens that are cytotoxic and concluded that the changes in expression of the *myc* and *fos* gene may be a mechanism by which chloroform induces regenerative cell proliferation.

The methylation state of genes in chloroform-induced liver tumors has been evaluated by Vorce and Goodman (1991) and Dees and Travis (1994). The methylation status of *ras* oncogenes was evaluated in liver tumors in male B6C3F1 mice that had received chloroform (200 mg/kg) via corn oil gavage twice weekly for 1 year (Vorce and Goodman, 1991). In all liver tumors examined in the treated and control groups, the Ha-ras was hypomethylated and occasional hypomethylation of the Ki-ras gene was also observed. The methylation status of the myc gene was not altered.

Exposures to chloroform at concentrations of 0.5%–2% (v/v) resulted in hypermethylation of the p53 protein in rat liver epithelial cells and in Saos-2 human sarcoma cells transfected with the gene for p53 (Dees and Travis, 1994). The authors noted that concurrent administration of other chemicals (phorbol myristate acetate, toluene, and benzene) resulted in greater hypermethylation and that each of these chemicals, including chloroform, has the capacity to stimulate protein kinase C. The authors proposed that the hypermethylation of p53 may have been due to the stimulation of protein kinase C, and that this may represent an alternative mode of action to chloroform carcinogenicity.

4.4.4. Studies of Interactions With Other Chemicals

Because of the importance of CYP2E1 in the metabolism and toxicity of chloroform, any chemical that induces the level of CYP2E1 activity is likely to also increase the toxicity of chloroform. A number of such chemicals are known, including many alcohols (including ethanol), aldehydes, aromatics, ethers, halogenated solvents, and heterocyclics (Ronis et al., 1996). The mechanisms by which these agents induce CYP2E1 appear to be complex and varied, including transcriptional, translational, and posttranslational mechanisms.

Other chemicals, specifically certain ketones, may potentiate the toxic effects of chloroform by mechanisms other than enzyme induction. For example, hepatic microsomal enzymes were induced to a greater extent with the insecticide mirex, when compared with its ketone analogue, chlordecone (Cianflone et al., 1980). However, the binding of chloroform to hepatic constituents was greater following pretreatment with chlordecone than with pretreatment with mirex. Hewitt et al. (1979) reported that pretreatment with chlordecone followed by exposure to chloroform resulted in an altered pattern of histological hepatic lesions, when

compared with the pattern of histological lesions observed following administration of the appropriate dose of chloroform that resulted in a comparable number of abnormal hepatocytes. Alcohols, which are metabolized to ketones, and other ketones have also been reported to potentiate the toxic effects of chloroform. Alloxan-induced diabetic rats, which were in a state of metabolic ketosis, were reported to be more sensitive to the effects of chloroform (Hanasono et al., 1975). The precise mode of action by which ketones potentiate chloroform toxicity is unclear; however, possible mechanisms include alterations in calcium pump activity (Moore and Ray, 1983) or an increased sensitivity to chloroform following exposure to ketones (Hewitt et al., 1990).

Other chemicals that may potentiate chloroform toxicity include dichloroacetic acid (Davis, 1992) and carbon tetrachloride (Borzelleca et al., 1990). In the study by Davis (1992), the administration of nontoxic doses of dichloroacetic acid to female Sprague-Dawley rats resulted in increased toxicity in the liver, based on increased plasma alanine aminotransferase levels, and in the kidney, based on increased blood urea nitrogen levels. Histological examinations of the liver and kidney were not conducted. The administration of trichloroacetic acid resulted in the potentiation of chloroform-induced nephrotoxicity. Borzelleca et al. (1990) reported greater evidence of hepatotoxicity, measured by increases in several serum enzymes, in rats that received concurrent oral administrations of carbon tetrachloride and chloroform, when compared with rats that received carbon tetrachloride or chloroform alone.

The toxicity of chloroform may be decreased by some chemicals as well. For example, hepatic and renal toxicity was decreased in rats that received concurrent oral administrations of chloroform and trichloroethylene, when compared with the toxicity observed in rats that received chloroform alone (Lilly, 1992). This effect was reported to be independent of the dosing vehicle.

4.5. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS AND MODE OF ACTION

The noncancer effects of chloroform exposure have been well characterized in numerous studies in animals. These studies reveal that oral or inhalation exposure to chloroform results in toxicity to the liver, kidney, and nasal epithelium. Data from studies of exposed humans are more limited, but support the conclusion that hepatotoxicity is the noncancer effect of chief concern following chronic exposure.

In laboratory animals, evidence of hepatic and renal damage is usually based on histological detection of fatty infiltration and degeneration, cellular necrosis, and cellular vacuolization, along with changes in serum enzyme levels, altered liver and kidney weight, and/or altered organ function. In some cases, evidence of cellular regeneration (presumably in response to antecedent cellular necrosis) can be detected using the labeling index even when frank cytotoxicity is not readily apparent.

Exposure to chloroform during pregnancy can also result in reproductive or developmental toxicity (U.S. EPA, 1998c). However, available studies indicate that these effects occur at the same or higher doses as those that cause effects on the dam (Thompson et al., 1974, 1988; Ruddick et al., 1983; Baeder and Hoffman, 1988, 1991; Schwetz et al., 1974), suggesting

that most of the effects are secondary to maternal toxicity. No studies were located that demonstrate that the fetus is more sensitive to chloroform toxicity than the mother.

There is strong evidence that the toxicity of chloroform is a result of the metabolism of the parent to toxic intermediates. This conclusion is based mainly on the observation that toxicity of chloroform is increased by chemicals that enhance metabolism and is reduced by chemicals that inhibit metabolism. Further, variations in chloroform toxicity between tissues and between species and genders tend to correlate with the level of metabolic enzymes and the metabolic capacity of the tissues and species.

Metabolism of chloroform may occur through one or both of two pathways: oxidative and reductive metabolism. Both pathways result in the formation of highly reactive metabolites: phosgene (oxidative) and dichloromethyl free radical (reductive) (U.S. EPA, 1994d). These reactive intermediates are capable of forming covalent adducts with cellular molecules (Pohl et al., 1980; Brown et al., 1974; Tyson et al., 1983), presumably resulting in impairment of cellular function and contributing to cell injury and death. In general, covalent binding of reactive metabolites to cellular molecules is highest in areas of the liver and kidney where cytotoxicity is greatest (e.g., Ilett et al., 1973). Free radicals produced via the reductive pathway may also induce lipid peroxidation; however, there are only limited data available to support this conclusion (U.S. EPA, 1994d).

The relative importance of the oxidative and reductive pathways has been investigated by several researchers, and the results suggest that metabolism occurs mainly via the oxidative pathway. First, reductive metabolism of chloroform is observed only in phenobarbital-induced animals or in tissues prepared from them, with negligible reducing activity observed in microsomes from uninduced animals (ILSI, 1997). Second, the reactive intermediates formed by oxidative metabolism bind to the polar heads of phospholipids, whereas the reductive metabolites bind to the fatty acid tails (ILSI, 1997). Thus, the pattern of lipid adducts formed can be used to distinguish which chloroform metabolic pathways are occurring under a specified test condition. Using this approach, Ade et al. (1994) showed that even under relatively low (2.6%) oxygen partial pressure (approximately average for the liver), more than 75% of the phospholipid binding was to the fatty acid heads, indicating metabolism was chiefly by the oxidative pathway (U.S. EPA, 1998c; ILSI, 1997). Third, addition of glutathione to the incubation system completely negated binding to liver microsomes, with only residual binding remaining in kidney microsomes (ILSI, 1997). This quenching by glutathione is expected for the products of oxidative but not reductive metabolism. Finally, the gender-specific pattern of renal toxicity observed in mice in vivo correlates with the level of adduct formation in proteins and lipids in vitro only under aerobic conditions. Taken together, these observations strongly support the conclusion that chloroform metabolism in vivo occurs primarily via the oxidative pathway except under special conditions of high chloroform doses in preinduced animals (ILSI, 1997; U.S. EPA, 1998c).

4.6. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION

4.6.1. Mode of Action

4.6.1.1. *Summary of Postulated Mode of Action*

Studies in humans are inadequate to determine if chloroform is carcinogenic. Studies in animals reveal that chloroform can cause an increased incidence of kidney tumors in male rats and an increased incidence of liver tumors in male and female mice. Tumors are produced only at dose levels that result in cytotoxicity. These induced tumor responses are postulated to be secondary to sustained or repeated cytotoxicity and secondary regenerative hyperplasia. Chloroform's carcinogenic effects in rodent liver and kidney are attributed to oxidative metabolism-mediated cytotoxicity in the target organs. Although chloroform undergoes both oxidative and reductive cytochrome P450-mediated metabolism, it is the oxidative (CYP2E1) metabolic pathway that predominates at low chloroform exposures. This oxidative pathway produces highly tissue-reactive metabolites (in particular phosgene) that lead to tissue injury and cell death. It is likely that the electrophilic metabolite phosgene causes cellular toxicity by reaction with tissue proteins and cellular macromolecules as well as phospholipids, glutathione, free cysteine, histidine, methionine, and tyrosine. The liver and kidney tumors induced by chloroform depend on persistent cytotoxic and regenerative cell proliferation responses. The persistent cell proliferation presumably would lead to higher probabilities of spontaneous cell mutation and subsequent cancer. The weight of the evidence indicates that a mutagenic mode of action via DNA reactivity is not a significant component of the chloroform carcinogenic process.

4.6.1.2. *Identification of Key Events*

There are essentially three key steps in the sequence of events that lead to chloroform-induced tumorigenesis in the liver and kidneys of rodents. The first step is oxidative metabolism of chloroform in the target organs, kidney and liver. Numerous binding and metabolism studies (as described in ILSI, 1997, and U.S. EPA, 1998c) support the conclusion that chloroform is metabolized by the oxidative cytochrome P450 (CYP2E1) pathway. This conclusion is supported by the study of Constan et al. (1999) in Sv/129 wild type, Sv/129 CYP2E1 null, and B6C3F1 mice. In the wild type of each strain, exposure to 90 ppm chloroform for 6 hours per day for 4 consecutive days resulted in severe hepatic and renal lesions along with increased cell proliferation. With the same exposure, neither the cytotoxicity nor cell proliferation occurred in the CYP2E1 null mouse or in the wild type of either strains treated with the P450 inhibitor 1-aminobenztriazole.

Available evidence indicates that metabolism by CYP2E1 predominates at low exposures and is rate-limiting to chloroform's carcinogenic potential. Reductive metabolism, if it occurs, can lead to free radicals and tissue damage, but this pathway is absent or minor under normal physiological conditions. The next key step is the resultant cytotoxicity and cell death caused by the oxidative metabolites (with phosgene as the significant toxic intermediate). Regenerative cell proliferation follows the hepatotoxicity and renal toxicity as measured by labeling index in mouse kidney and liver and rat kidney from chloroform-treated animals.

This increase in cell division can lead to an increased probability of cancer by one or both of two alternative modes of action. First, cells that are undergoing cell division are inherently more susceptible to initiation than are slowly growing or nondividing cells. This is because DNA undergoing replication is more exposed to nucleophilic attack than DNA that is covered with histones and arranged in nucleosomes (Ames and Gold, 1991a,b). Also, any gene damage that occurs in a cell undergoing division has less time to be repaired before mitosis than in a slowly growing cell, so a larger fraction of DNA alterations could be converted into mutations. Second, chemicals that promote cell division may convey a selective growth advantage to preexisting initiated cells in comparison with normal cells, thereby facilitating clonal expansion of initiated cells. This could occur because initiated cells are more responsive than normal cells to growth stimuli, because they are less susceptible to the toxicity of the chemical, or because they are less susceptible to endogenous regulatory signals that trigger programmed cell death (apoptosis). In any case, the ratio of cell birth to cell death of initiated cells increases compared with normal cells, leading to increased likelihood that a clone of initiated cells will form and survive. A key characteristic of this mode of action is that the effect is reversible: the clones of induced cells will tend to regress if the promoter (mitogen, cytotoxicant) is withdrawn (Pitot et al., 1987; Schulte-Hermann et al., 1993).

4.6.1.3. *Strength, Consistency, Specificity of Association*

Table 3 summarizes information on the correlation between the occurrence of statistically significant increases in cancer prevalence and evidence of cytotoxicity and regenerative hyperplasia (mainly in the form of increased labeling index) in animals exposed to chloroform. Inspection of Table 3 reveals two main points:

- There are numerous cases where exposure to chloroform causes an increase in the LI without any observable increase in cancer incidence. These data indicate that chloroform exposures that are adequate to cause cytotoxicity and regenerative cell proliferation do not always lead to cancer.

- There are no cases in which a tumorigenic response has been observed where evidence of cell regeneration is not also observed at the same or lower dose as that which caused an increase in tumors. This consistency of evidence (i.e., cell regeneration is detected in all cases of tumorigenicity) is strong evidence supporting the conclusion that cell regeneration is a mandatory precursor for tumorigenicity.

Evidence for a link between sustained cytotoxicity/regenerative hyperplasia and cancer is strongest in the kidney. In male Osborne Mendel rats exposed to chloroform in water for 2 years (Jorgenson et al., 1985), a statistically significant increase in renal tumors was observed at a concentration of 1,800 ppm (160 mg/kg/day). A reanalysis of the histopathological slides from this study (Hard et al., 2000) revealed evidence for sustained cytotoxicity and cell proliferation in the kidney at exposures of 900 ppm (81 mg/kg/day) or higher. Likewise, in BDF_1 mice exposed

Table 3. Correlation of carcinogenicity and regenerative cell hyperplasia

Target tissue	Test species	Gender	Exposure route	Cancer bioassay				Evidence of cell regeneration			
				Strain	Effect	Dose level[a]	Reference	Strain	Effect	Dose level	Reference
Liver	Mouse	Male	Gavage	B6C3F1	+	138	NCI, 1976	B6C3F1	+ (LI)	34 (4 days)	Larson et al., 1994c
			Inhalation	BDF1	-	90 ppm	Nagano et al., 1998	BDF1	+ (LI)	90 ppm (7 wks)	Templin et al., 1998
		Female	Gavage	B6C3F1	+	238	NCI, 1976	B6C3F1	+ (LI)	238 (4 days)	Larson et al., 1994b
			Drinking water	B6C3F1	-	263	Jorgenson et al., 1985	B6C3F1	- (LI)	329 (4d-3wks)	Larson et al., 1994b
			Inhalation	BDF1	-	90 ppm	Nagano et al., 1998	BDF1	+ (LI)	90 ppm (3-13 wks)	Templin et al., 1998
	Rat	Male	Gavage	OM	-	180	NCI, 1976	OM	- (LI)	477 (1 day)	Templin et al., 1996b
			Drinking water	OM	-	160	Jorgenson et al., 1985	F-344	- (LI)	106 (4d-3wks)	Larson et al., 1995a
			Inhalation	F-344	-	90 ppm	Nagano et al., 1998.,	F-344	+ (LI)	300 ppm (4d-13 wks)	Templin et al., 1996a
		Female	Gavage	OM	-	200	NCI, 1976	F-344	+ (LI)	100 (4d-3 wks)	Larson et al., 1995b
			Inhalation	F-344	-	100 ppm	Nagano et al., 1998	F-344	+ (LI)	300 ppm (4d-13 wks)	Templin et al., 1996a
Kidney	Mouse	Male	Gavage	B6C3F1	-	277	NCI, 1976	B6C3F1	+ (LI)	34 (4 days)	Larson et al., 1994c
			Inhalation	BDF1	+	30 ppm	Nagano et al., 1998	BDF1	+ (LI)	30 ppm (7-13 wks)	Templin et al., 1998
		Female	Gavage	B6C3F1	-	477	NCI, 1976	B6C3F1	+ (LI)	477 (4 days)	Larson et al., 1994b
			Drinking water	B6C3F1	-	263	Jorgenson et al., 1985	B6C3F1	+ (LI)	43 (3 wks)	Larson et al., 1994b
			Inhalation	BDF1	-	90 ppm	Nagano et al., 1998	BDF1	- (LI)	90 ppm (3-13 wks)	Templin et al., 1998
	Rat	Male	Gavage	OM	+	180	NCI, 1976	OM	+ (LI)	10 (1 day)	Templin et al., 1996b
			Drinking water	OM	+	160	Jorgenson et al., 1985	F-344 OM	+ (HP) + (HP)	17 (3 wks) 81 (6-24 mo)	Larson et al., 1995a Hard et al., 2000
			Inhalation	F-344	-	90 ppm	Nagano et al., 1998	F-344	+ (LI)	30 ppm (3 wks)	Templin et al., 1996a
		Female	Gavage	OM	-	200	NCI, 1976	F-344	+ (LI)	100 (4d-3 wks)	Larson et al., 1995b
			Inhalation	F344	-	90 ppm	Nagano et al., 1998	F-344	+ (LI)	30 ppm (13 wks)	Templin et al., 1996a

[a] All doses oral doses are expressed as mg/kg/day. All inhalation exposures are expressed as ppm in air.
LI = labeling Index
HP = histopathology

to chloroform by inhalation at 5, 30, or 90 ppm for 6 h/day, 5 days/week (Nagano et al., 1998), increased incidence of renal tumors was observed in male mice at the two higher doses while females showed no significant tumor response. Templin et al. (1998) duplicated this exposure regimen in order to study whether the treatment caused cytotoxicity and regenerative hyperplasia. These authors observed cytotoxicity and hyperplasia in the kidneys of male mice exposed to 30 or 90 ppm throughout a 90-day exposure period, but not in females. This observation is consistent with the hypothesis that sustained cytotoxicity and regenerative hyperplasia are key events in the neoplastic response of the kidney to chloroform.

Available data also indicate that cytotoxicity and regenerative hyperplasia are required for liver cancer, although the strength of this conclusion is somewhat limited because most of the observations are based on short-term rather than long-term histological or labeling index measurements. For example, in the B6C3F1 mouse, corn oil gavage (bolus dosing) at the same doses that resulted in liver tumors in the study by NCI (1976) also caused hepatic cytolethality and a cell proliferative response at 4 days and 3 weeks (Larson et al. 1994b,c). Similarly, exposure of female B6C3F1 mice to chloroform in drinking water at levels that did not induce liver tumors (Jorgenson et al., 1985) also did not induce hepatic cytolethality or cell proliferation at 4 days or 3 weeks (Larson et al., 1994b). This consistency of the data (i.e., evidence of cytolethality and/or regenerative hyperplasia is always observed in cases of increased liver tumors) supports the conclusion that this liver cancer also occurs via a mode of action involving regenerative hyperplasia.

4.6.1.4. *Dose-Response Relationship*

Chloroform-induced liver tumors in mice are only seen after bolus corn oil dosing. Mouse liver tumors are not found following administration by other routes (drinking water and inhalation). Rat liver tumors are not induced by chloroform following either drinking water or corn oil gavage administration. Kidney tumors are found in mice exposed to chloroform via inhalation or in toothpaste preparations, and in rats when exposed via drinking water or corn oil gavage. Kidney and liver tumors develop only at doses that cause persistent cytotoxicity and regenerative proliferation, regardless of route of exposure or dosing regime. The dose-response curves for the cytotoxicity and cell proliferation responses are nonlinear. All key events and tumor effects depend on the dose rate, as shown by the difference in oil gavage versus drinking water administration (ILSI, 1997; U.S. EPA, 1998c).

4.6.1.5. *Temporal Relationship*

As noted above, there is very strong evidence from short-term and long-term histological and labeling index studies in mice and rats that cytotoxicity and cell proliferation always precede increased kidney or liver tumor effects in long-term bioassays. For example, a reevaluation of serial sacrifice data from the chloroform 2-year drinking water bioassay in Osborne-Mendel rats revealed a linkage between toxicity in the renal tubules and tumor development and showed that renal toxicity preceded tumor development (Hard and Wolf, 1999; Hard et al., 2000).

4.6.1.6. *Biological Plausibility and Coherence*

The theory that sustained cell proliferation to replace cells killed by toxicity, viral, or other insult such as physical abrasion of tissues can be a significant risk factor for cancer is plausible and generally accepted (Correa, 1996). It is logical to deduce that sustained cytotoxicity and regenerative cell proliferation may result in a greater likelihood of spontaneous mutations being perpetuated, with the possibility of one or more of these resulting in uncontrolled growth. It may also be that continuous stimulus of proliferation by growth factors involved in inflammatory responses increases the probability that damaged cells may slip through cell cycle checkpoints carrying DNA alterations that would otherwise be repaired. Current views of cancer processes support both possibilities. There are no data on chloroform that allow the events that occur during cell proliferation to be directly observed. A high proliferation rate alone is not assumed to cause cancer; tissues with naturally high rates of turnover do not necessarily have high rates of cancer, and tissue toxicity in animal studies does not invariably lead to cancer. Nevertheless, regenerative proliferation associated with persistent cytotoxicity appears to be a risk factor of consequence.

4.6.1.7. *Role of Mutagenicity*

The question whether chloroform or a metabolite is mutagenic has been tested extensively across different phylogenetic orders (i.e., bacterial, eukaryotic, and mammalian systems). Predominately negative results are reported in all test systems, with no pattern of mutagenicity seen in any one system considered to be a competent predictor. Positive results appear sporadically in the database, but are outnumbered by negative results in other tests in the same system. ILSI (1997) considered results from 40 tests by the quantitative weight-of-evidence method for heterogeneous genetic toxicology databases from the International Commission for Protection against Environmental Mutagens and Carcinogens (ICEMC) (Lohman et al., 1992). This method scores relative DNA reactivity with a maximum positive score being +100, and maximum negative of –100. The maximum positive score obtained among 100 chemical databases has been +49.7 (triazaquone) and the maximum negative has been –27.7. The score for chloroform was –14.3.

Testing of chloroform in the p53 heterozygous knockout mouse shows no tumor effect (Gollapudi et al., 1999). Heterozygous p53 males were dosed up to 140 mg/kg and females up to 240 mg/kg via corn oil gavage for 13 weeks. This model is known to respond to most mutagenic carcinogens.

Products of oxidative and reductive metabolism of chloroform are highly reactive. Such species are unstable and will likely react with cytoplasmic molecules before reaching nuclear DNA. Such reactive species (e.g., phosgene) have not been evaluated separately for genetic toxicity, and because of reactivity would not be amenable to study and would not likely be able to transport from the cellular site of production to the nucleus.

Comparative examination of both oxidative and reductive metabolism for structural analogues and chloroform has revealed that carbon tetrachloride, which is largely metabolized to a free radical via the reductive pathway, results in cell toxicity, not mutagenicity. Moreover, chloroform and carbon tetrachloride show very different patterns of liver toxicity (i.e., carbon

tetrachloride's toxicity is more consistent with free radical production and chloroform's is not). For methylene chloride, glutathione conjugation results in mutagenic metabolites. When rat glutathione transferase gene copies are introduced into *Salmonella*, bromodichloromethane produces mutagenic metabolites; the fact that chloroform in this system did so only marginally and only at high toxic doses (Pegram et al., 1997) provides support for a conclusion that the reductive pathway does not contribute to chloroform's toxicity and carcinogenicity.

In initiation-promotion studies, chloroform at the highest test dose of the drinking water bioassay, does not promote development of hepatic lesions in rats or two strains of mice, nor does it initiate or act as a co-carcinogen. Administered in oil, chloroform was a promoter in the rat liver in initiation-promotion protocols. These results are more consistent with the postulated mode of action than with any mutagenic potential.

4.6.1.8. *Conclusion Regarding Cancer Mode of Action*

The weight of the evidence supports the conclusion that chloroform-induced tumors in liver and kidney are only produced at dose levels that result in repeated or sustained cytotoxicity and regenerative cell proliferation. A wide range of evidence across different species, sexes, and routes of exposure implicates oxidative CYP2E1 metabolism leading to persistent cytotoxicity and regenerative cell proliferation as events that precede and are associated with tumor formation. The cytochrome P450 oxidative metabolism that leads to oxidative damage and ensuing cell growth, involving basic tissue responses to cellular toxicity and death, is common to humans and rodents. No data exist that indicate the mode of action observed in rodents is not also likely to apply to humans.

Available data on the mutagenic and genotoxic potential of chloroform are mixed, but the majority of tests are negative, and some of the positive results are observed only at extreme exposure conditions. Thus, the weight of the evidence indicates that chloroform is not a strong mutagen and that neither chloroform nor its metabolites readily bind to DNA. Based on these results and the results of studies that evaluated other endpoints of genotoxicity, it seems likely that even though a role for mutagenicity cannot be excluded with certainty, chloroform does not produce carcinogenic effects primarily by a specific genotoxic mechanism.

4.6.2. Weight of Evidence

Under the 1986 U.S. EPA Guidelines for Carcinogen Risk Assessment, chloroform has been classified as Group B2, *probable human carcinogen*, based on sufficient evidence of carcinogenicity in animals (U.S. EPA, 1998d).

Under the Proposed Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1996a, Federal Register 61[79]:17960-18011; U.S. EPA, 1999), chloroform is *likely to be carcinogenic to humans* by all routes of exposure under dose conditions that lead to cytotoxicity and regenerative hyperplasia in susceptible tissues (U.S. EPA, 1998c,d). Chloroform is *not likely to be carcinogenic to humans* by all routes of exposure at dose levels that do not cause cytotoxicity and cell regeneration. This weight-of-evidence conclusion is based on: (1) Observations in animals exposed by both oral and inhalation pathways indicate that sustained or repeated cytotoxicity with secondary regenerative hyperplasia precedes, and is probably required for,

hepatic and renal neoplasia; (2) there are no epidemiological data specific to chloroform and, at most, equivocal epidemiological data related to drinking water exposures that cannot necessarily be attributed to chloroform amongst multiple other disinfection byproducts; and (3) the weight of evidence of the genotoxicity data on chloroform supports a conclusion that chloroform is not strongly mutagenic, and that genotoxicity is not likely to be the predominant mode of action underlying the carcinogenic potential of chloroform. Although no cancer data exist for exposures via the dermal pathway, the weight-of-evidence conclusion is considered to be applicable to this pathway as well, because chloroform absorbed through the skin and into the blood is expected to be metabolized and to cause toxicity in much the same way as chloroform absorbed by other exposure routes.

4.7. SUSCEPTIBLE POPULATIONS

A susceptible population is any group of people who may be at increased risk of experiencing an adverse effect from an environmental chemical compared with other members of the population. In general, two factors may contribute to increased susceptibility: higher than average risk of exposure, and higher than average adverse response per unit exposure. Individuals may have higher exposure to a chemical because they reside or work in an area with elevated concentrations in the environment, or because they have higher than average intake of contaminated environmental media. For example, children often have higher intakes per unit body weight of water, air, and food than do adults, and this may contribute to an increased exposure rate during childhood compared to adulthood. Individuals may have a higher than average adverse response per unit exposure for a number of reasons, including increased absorption, decreased excretion, higher or lower metabolism (depending on whether metabolism increases or decreases toxicity), decreased cellular defense and repair mechanisms, etc. The following section discusses available data on whether there are any subpopulations that may be especially susceptible to the adverse effects of chloroform.

4.7.1. Possible Childhood Susceptibility

The central questions asked in a mode of action analysis are, 1) whether the standard assumption that a mode of action observed in animals is relevant to humans holds true in a particular case, and 2) what the nature of the mode of action implies about the shape of the dose response relationship. In the case of chloroform the conclusions have been that the rodent mode of action can be assumed to be relevant to humans and that a nonlinear approach is most appropriate. The next question is whether the data lead one to anticipate similarities or differences in response by sex or age.

Ideally, one would have adequate data to compare each of the key events of chloroform toxicity and subsequent carcinogenicity in tissues of adults with those of the developing fetus and young. This kind of information is currently not to be found. In the absence of data on the fetus and young specific to chloroform, an evaluation is made as to whether a cogent biological rationale exists for determining that the postulated mode of action is applicable to children (EPA, 1999). There is no suggestion from available studies of chloroform to indicate that children or fetuses would be qualitatively more sensitive to its effects than adults. The developing organism would not be expected to be particularly sensitive to cytotoxic agents at minimally toxic levels

because cell division is proceeding rapidly and repair capacity at the molecular and cellular level is high. This is reflected by the relatively low incidence of spontaneous tumors in developing and young organisms. Moreover, the reproductive and developmental studies available, while they have limitations, show that fetal effects are seen only at doses at which maternal toxicity is evident. Research would be needed to further explore whether there are circumstances in which this relationship does not hold. Research would also be needed to discover whether there is some other mode of action, not seen in rodents, that might be possible. Presently, there are no clues from in vivo or in vitro studies as to what alternative mode of action might be considered. In keeping with traditional toxicologic evaluations, chloroform has been tested in lifetime studies with high level doses to provide maximal opportunities for toxicologic effects to manifest themselves in multiple tissues and organs through multiple mechanisms. In the absence of data to the contrary, this approach is considered to provide evidence for lack of potential for significant response, other than those noted, even for sensitive individuals and life stages.

The mode of action analyzed as well as all other potential modes of action identified required that chloroform be metabolized by cytochrome P450 (CYP2E1) (SAB (2000), p.2). When this is considered along with the comparison of this enzyme activity between adults and the young there is confidence in assuming similarity in response among life stages. Further research on the processes of cell injury, death and regeneration would increase this confidence by addressing any uncertainty about potential quantitative similarity. The literature does not reveal any such quantitative data at present.

Given the above, it is reasonable to assume that: 1) The reactive metabolite inside the cell should have similar effects by reacting with and disrupting macromolecules in the cells of fetuses, children and adults, 2) Cell necrosis and reparative replication are not likely to be qualitatively different in various stages in life, 3) Cancer risk to the fetus or children would be a function of cytotoxic injury, like in adults, and protecting these life stages from sufficient cytotoxicity to elicit this response should protect against cancer risks. Further research would be needed to assess whether there are significant quantitative differences between life stages which have not yet been elucidated.

It can be noted that if data indicated that it were appropriate to apply a linear approach to part of a lifetime, such as the first 3 years of life, the resulting risk would be represented by a small increment of the total dose per body weight over a lifetime since most of a 70 year life is at an adult body weight. When this total is divided by 70 years to derive the lifetime average daily dose, the small increment of early dose does not significantly increase risk. The RfD (in mg/kg/dy) estimated for chloroform's cancer effect would fall at about 4.2×10^{-5} on a line of linear extrapolation if dose were at the RfD level for a lifetime. If one assumed a higher dose (in mg/kg/dy) for the first three years of life, e.g., three times, the added risk would be 4.21×10^{-5} for a lifetime.

4.7.1.1. *U.S. Incidence of Kidney and Liver Cancer in Children*

The National Cancer Institute's SEER Pediatric Monograph on the cancer incidence and survival among children and adolescents reports that Wilms' tumor (a tumor due to a germ cell mutation) accounts for 95% of the renal cancers in children and those younger than 20 (Reis et al., 1999). Renal cancer accounts for about 6.3% of cancer diagnoses in children younger than 15

an 4.4% of diagnoses in those younger than 20. As to risk factors the report says in part: "A small proportion of Wilms' tumor cases appear to be heritable including those patients with bilateral tumors, those occurring in association with aniridia and other congenital disorders, and those few cases arising in the small number of families with one or more additional cases of Wilms' tumor in close family members....Most of the analytical and epidemiologic investigations of childhood renal cancer have focused on Wilms' tumor, and very little is known about risk factors for childhood renal carcinoma or the other rarer childhood renal cancer subtypes. Several epidemiological studies have investigated occupational, environmental, and lifestyle characteristics as potential risk factors for Wilms' tumor. A number of parental and childhood exposures have been found to be associated with an increased risk of Wilms' tumor. Most of these associations have not been replicated in multiple high quality studies. However, some warrant further evaluation including paternal occupational exposures, pesticide exposure, and certain maternal exposures during pregnancy." Liver cancer is rare in children; 100-150 children younger than 20 are diagnosed with liver cancer yearly, about 1% of childhood cancers. Hepatoblastoma, a congenital cancer, is the most frequent liver neoplasm in infancy to 4 years, and thus is not related to adult onset of liver cancer. Less frequent is hepatocarcinoma, which increases in proportion with age and is the prevalent adult tumor type. Recent studies have suggested an association of hepatoblastoma with prematurity and its treatment. For both liver and kidney cancer, there are studies reporting evidence of association with specific parental occupational exposure to metals and organic chemicals and with maternal medication or other exposures; as yet, no identification of a causal agent or agents has been made.

A potential limitation to the use of cancer incidence data to assess the relative sensitivity of children versus adults is that exposures during childhood may not result in a neoplastic response until later in life. Thus, the low incidence of renal and hepatic tumors in children should be interpreted as evidence that is consistent with (but not proof of) the idea that children are not more susceptible than adults. Indeed, there are no direct data that indicate whether exposures to chloroform during childhood are or are not associated with increased risk of cancer later in life.

4.7.1.2. *Liver Toxicity in Younger Versus Older Rodents*

In a two-generation study (NTP, 1988), CD-1 (ICR)BR mice were exposed to chloroform in utero, during lactation, and then by gavage as young mice through "young" adulthood. The only liver effect observed was mild to moderate liver histopathology (degeneration of centrilobular hepatocytes, accompanied by occasional single-cell necrosis) in females at 41 mg/kg/day, the only dose at which systemic effects were evaluated. Thus, the only dose tested in this study, 41 mg/kg/day, was a LOAEL for liver histopathology (U.S. EPA, 1998c). No effects of chloroform on reproductive function were identified (NTP, 1988). Oral developmental toxicity studies have found decreased fetal weight (Thompson et al., 1974), and inhalation developmental studies have found an increased incidence of delayed ossification in Wistar rats (Baeder and Hofmann, 1991), but these effects occurred at doses above those causing hepatotoxicity.

4.7.1.3. *Metabolism of Chloroform in Fetuses, Infants, and Children Compared with Adults—Implications for Quantitative Dose-Response Relationship*

Metabolism of chloroform is essential to its toxicity (U.S. EPA, 1998c). Moreover, metabolism by cytochrome P450 CYP2E1 is required for toxicity to both liver and kidney of B6C3F1 and Sv/129 male mice (Constan et al., 1999). Because of the role of CYP2E1 in chloroform's mode of carcinogenic action, it is important to evaluate CYP2E1 activity in tissues of the young compared with adults to determine whether the young might respond at a lower dose than adults.

Most studies on CYP2E1 levels in humans indicate that this enzyme is expressed in human adults but not in human fetuses, even when measured using sensitive assays (reviewed in Hakkola et al., 1998). In these studies, levels of both CYP2E1 protein and of the associated enzyme activity were undetectable before birth, but rose rapidly shortly after birth. For example, Vieira et al. (1996) found that CYP2E1 protein could not be detected immunochemically in fetal human liver, and there was only minimal evidence of CYP2E1 mRNA or CYP2E1 activity in fetal liver microsomes. (The difference in assay results may be due to differences in sensitivity, or to cross-reaction of CYP1A1 activity, but the authors noted that "it is generally assumed that CYP1A1 is not expressed in appreciable amounts in human livers.") The authors found, however, that CYP2E1 protein levels rise rapidly in the first few hours after birth, with a slow increase in protein levels and in CYP2E1 RNA levels during childhood. However, a few studies indicate CYP2E1 is expressed in fetal liver or cephalic tissue (Boutelet-Bochan et al., 1997; Carpenter et al., 1996). Boutelet-Bochan et al. (1997) detected low levels of CYP2E1 mRNA transcription in human fetal brains (gestation days 52–117, or 7–17 weeks), and levels tended to increase with gestational age. Transcription was detected with a very sensitive assay (reverse transcriptase-polymerase chain reaction, RT-PCR) or the moderately sensitive RNase protection assay. Transcription in fetal liver was much lower and was detectable in only two of six samples. Also using the RNase technique, Carpenter et al. (1996) found transcription of CYP2E1 mRNA in the liver of human fetuses at 19–24 weeks gestation, but not at 10 weeks gestation. Fetal liver microsomes could metabolize the CYP2E1 substrate ethanol, but at a rate only 12%–27% of adult liver microsomes. Most of the observed activity was specific to CYP2E1, as it was inhibited by an anti-CYP2E1 antibody. Like adult hepatocytes, fetal hepatocytes exposed to ethanol had induced levels of CYP2E1. Thus, maternal exposures to ethanol and other inducers of CYP2E1 might increase fetal levels of the enzyme and hence might increase sensitivity to chloroform exposures that occurred during or after birth.

Studies in humans indicate CYP2E1 enzymic activity in human fetuses is either absent or low compared with that in adult tissues. However, the enzyme is rapidly induced upon birth, although the amount of CYP2E1 at birth may be less than that present in the adult. Given that metabolism of chloroform is necessary to its carcinogenicity, the data on CYP2E1 and considering that the amount of this enzyme's activity appears to be less than adult amounts in the fetus and less or equal in children depending on age, the data provide no evidence to suggest that fetuses or children are more susceptible than adults due to this metabolic activity.

Studies in animals on the developmental regulation of CYP2E1 provide uniform evidence of the rapid induction of this gene soon after birth (Song et al., 1986; Umeno et al., 1988; Schenkman et al., 1989; Ueno and Gonzalez, 1990). The idea suggested by some scientists that the enzyme activity peaks before weaning with a gradual decrease to adult levels has not been consistently reported in the three studies that compared expression over this period of time.

For example, Schenkman et al. (1989) indicate that CYP2E1 protein is present in low levels in neonates, rises to a peak level at age 2 weeks, and subsequently decreases to adult levels by puberty. Analysis of protein levels quantified from western blots showed a maximum at 2 weeks with decreasing levels at 4 and 12 weeks. The protein level at 12 weeks was approximately 50% of the level at 2 weeks. The authors did not provide a statistical analysis of this result, but it appears from the error bars that the 2-week and 12-week levels (but not 4-week levels) were significantly different.

Song et al. (1986) conducted a similar analysis and reported a rapid transcriptional induction of CYP2E1 within 1 week following birth that remained elevated throughout 12 weeks. The authors did not quantify the western blots, but visual inspection indicates a small decline in protein levels by 12 weeks. However, in this same study, enzyme activity gradually increased over time, reaching a maximum at adulthood.

Ueno and Gonzalez (1990) showed that extracts from 3-day-old and 12-week-old rat liver, but not fetal or newborn rat liver, were able to generate significant CYP2E1 transcription in vitro. The ability of the extract to drive transcription of CYP2E1 was slightly greater at 12 weeks.

Taken together, these animal studies do not provide conclusive evidence of an early period of increased enzymatic activity in young animals when compared with adults. While the animal data remain unclear regarding the potential for a period of increased CYP2E1 activity above that in the adult, for humans, a gradual increase of CYP2E1 activity throughout childhood with a maximum level at adulthood, as described by Hakkola et al. (1998), appears to be the most likely situation.

4.7.1.4. *Conclusion Regarding Risks to Children*

The evidence provides no basis to conclude that the mode of action of chloroform (CYP2E1-dependent generation of phosgene leading to cytotoxicity in liver and kidney) would differ between children and adults. Neither the fetus, nor the child appears to more sensitive based on level of CYP2E1 activity. Studies in humans indicates CYP2E1 enzymic activity in human fetuses is either absent or low compared with that in adult tissues. However, the enzyme is rapidly induced upon birth, although the amount of CYP2E1 at birth may be less than that present in the adult.

4.7.2. Possible Gender Differences

Cancer statistics for the United States (Wingo et al., 1995) indicate that liver cancer incidence is similar in males and females, with an annual incidence rate in 1995 of about 0.007%. Alcohol consumption and viral infection are the two risk factors most often cited. Kidney cancer occurs with an annual incidence of about 0.01% and is about 50% more common in men than in women. Most kidney tumors are renal adenocarcinomas. The risk factors cited have been smoking, radiation, obesity, and pharmaceuticals, and kidney cancer does not appear to be associated with occupational exposures.

Studies in animals reveal gender-specific differences in the renal toxicity or carcinogenicity of chloroform. These differences are generally thought to be a result of gender-specific differences in the level of cytochrome P450 enzymes responsible for the metabolism of chloroform. For example, male mice are more sensitive to the nephrotoxic effects of chloroform than are female mice (Culliford and Hewitt, 1957; Eschenbrenner and Miller, 1945), and the metabolism of chloroform in the kidneys of male mice is greater than that in female mice (Taylor et al., 1974). The concentration of CYP2E1 (and hence the toxicity of chloroform) is induced by testosterone (ILSI, 1997), and as a consequence renal toxicity following chloroform administration is low in female mice and marked in male mice. This is supported by the finding that testosterone treatment of female mice increases renal toxicity following administration of chloroform (ILSI, 1997).

Gender-specific differences in hepatic toxicity also have been reported in rats and mice. In female rats, evidence of liver toxicity or carcinogenicity has been observed following administration of chloroform, whereas similar responses were not observed in males administered similar concentrations of chloroform. In the study conducted by Tumasonis et al. (1987), an increase in the incidence of hepatic adenomas was observed in female rats (0/18 in controls versus 10/40 in chloroform-treated animals) administered chloroform in drinking water. No increase in the incidence of hepatic lesions was observed in male rats, even though available data indicate that the CYP2E1 isozyme is expressed in higher amounts in the male rat than in the female rat (Ronis et al., 1996). In mice, a similar increase in the incidence of liver lesions (degeneration of centrolobular hepatocytes, accompanied by occasional single-cell necrosis) was observed in females administered chloroform by gavage in corn oil, with no increase in the incidence of this lesion observed in male mice. The reason for the gender-specific difference in hepatic toxicity is not clear, but might be gender-specific differences in hepatocellular protective mechanisms (e.g., glutathione levels).

These studies provide evidence of gender-specific susceptibilities in experimental animals. Whether gender-specific differences in chloroform toxicity also occur in human populations is currently unknown.

4.7.3. Other Factors That May Increase Susceptibility

As noted earlier, CYP2E1 is induced by a wide variety of alcohols, ketones, and other chemicals, so people exposed to these substances may have higher enzyme levels (and hence a greater capacity to metabolize chloroform) than the average individual. This includes people with excess intake of ethanol.

Dietary status may also influence chloroform metabolism. Wang et al. (1995) reported that overnight food deprivation resulted in a threefold increase in the metabolism and toxicity of chloroform compared to fed animals. Cytochrome P450 levels were not significantly increased by fasting, suggesting that the effect may not have been mediated by enzyme induction.

5. DOSE-RESPONSE ASSESSMENTS

5.1. ORAL REFERENCE DOSE

Data on the noncancer effects of chloroform were used to estimate RfD values using two different approaches: the traditional NOAEL-LOAEL approach and the benchmark dose (BMD) modeling approach (U.S. EPA, 1995).

5.1.1. NOAEL-LOAEL Approach

5.1.1.1. *Data Summary and Choice of Principal Study*

Table 4 summarizes oral exposure studies that were considered as candidates for the derivation of the chronic oral RfD for chloroform. No reliable long-term oral exposure studies in humans were located, so only studies in animals were evaluated. In order to ensure that the most sensitive endpoint was selected, the list of candidate studies includes not only longer term studies of liver and kidney toxicity, but also shorter term studies of reproductive and developmental effects, as well as several short-term studies based on labeling index in liver or kidney.

Many studies indicate that liver toxicity is the most sensitive non cancer endpoint following chronic oral exposure of animals to chloroform. Effects on the liver can be detected in a number of ways, including increased liver fat and/or histological evidence of hepatic cytotoxicity. The principal study selected to derive the RfD was the report by Heywood et al. (1979), in which there was an increase in the incidence of moderate to marked hepatic fatty cysts in dogs. This study was selected because it identifies the lowest LOAEL, and because it is also the longest duration study (7.5 years). The lesions observed in this study were characterized by aggregations of vacuolated histiocytes. Although fatty cysts were observed in the control group as well as all treated groups, both the size and severity of these lesions were significantly increased in treated animals. Although fatty cysts in liver are not a common endpoint, fat accumulation in the liver is a common and characteristic effect of chloroform exposure (see Roe et al., 1979; Jorgenson and Rushbrook, 1980; Jorgenson et al., 1982; DeAngelo et al., 1995; Thompson et al., 1974), supporting the view that fatty cysts are an authentic and toxicologically relevant endpoint. This is supported by the observation that chloroform exposure caused a sustained and dose-responsive increase in SGPT levels in the exposed animals, indicative of low-level hepatocytotoxicity.

5.1.1.2. *No-Observed-Adverse-Effect Level and Lowest-Observed-Adverse Effect Level*

The study by Heywood et al. (1979) identified a LOAEL of 15 mg/kg/day, based on the increase in the number and severity of hepatic fatty cysts in dogs. Because this was the lowest dose tested, a NOAEL was not identified.

5.1.1.3. *Derivation of the Oral Reference Dose*

The LOAEL of 15 mg/kg/day identified by Heywood et al. (1979) is used to derive a chronic oral RfD for chloroform as follows:

Table 4. Summary of oral noncancer studies in animals

Endpoint	Reference	Species/strain	Gender	Vehicle	Duration	NOAEL	LOAEL	Basis
Systemic (Body weight, liver and/or renal effects)	Roe et al., 1979	Mouse: ICI, C57BL, CBA, CFI	M,F	Toothpaste	80 wks	17	60	Moderate/severe fatty liver
	Palmer et al., 1979	Rat: Sprague-Dawley	M,F	Toothpaste	80 wks	—	60	Decreased weight gain in males and females (10%), increased liver weight in females
	Heywood et al., 1979	Dog: Beagle	M,F	Toothpaste	7.5 yr	—	15	Increased incidence and severity of fatty cysts in liver, increased SGPT
	Jorgenson et al., 1980	Mouse: B6C3F1	F	Drinking water	30-90 d	145	290	Increased liver fat
	Jorgenson et al., 1982	Mouse: B6C3F1	F	Drinking water	3-6 mo	34	65-130	Increased liver fat
	Jorgenson et al., 1982	Rat: Osborne-Mendel	M	Drinking water	1-6 mo	160	—	No increase in liver fat
	Jorgenson et al., 1985	Rat: Osborne-Mendel	M	Drinking water	104 wks	38	81	Histological evidence of renal tubular cytotoxicity
	Hard et al., 2000	Rat: Osborne-Mendel	M	Drinking water	6-24 mo	38	81	Histological evidence of renal tubular cytotoxicity
	Larson et al., 1994b	Mouse: B6C3F1	F	DW	3 wks	16	43	Small increase in LI in kidney (no effect in liver)
	Larson et al., 1995b	Rat: F344	F	Corn oil	3 wks	34	100	Increased LI in liver, kidney, nasal turbinates
	Bull et al., 1986	Mouse: B6C3F1	M,F	Corn oil	90 d	—	270	Diffuse hepatic degeneration, mild cirrhosis
	Bull et al., 1986	Mouse: B6C3F1	M,F	Emulphor	90 d	270	—	No hepatic toxicity observed
Reproductive or developmental effects	Thompson et al., 1974	Rat: Sprague-Dawley	F	Corn oil	6-15 of gest.	20 50	50 126	Dams (decreased wt, mild fatty changes in liver) Fetus (decreased weight)
	Thompson et al., 1974	Rabbit: Dutch-belted	F	Corn oil	6-18 of gest.	35	50	Maternal toxicity (no fetotoxicity)
	NTP, 1988	Mouse: CD-1 (ICR) BR	M,F	Corn oil	F1 generation	—	41	Liver histopathology in females
	Ruddick et al., 1983	Rat: Sprague-Dawley	F	Corn oil	6-15 of gest.	—	100	Decreased weight in dams and fetuses

$$\text{RfD} = \frac{15\ \text{mg/kg/day} \cdot (6\ \text{days}/7\ \text{days})}{1{,}000} = 1\text{E}-02\ \ \text{mg/kg/day}$$

where:

15 mg/kg/day =	LOAEL identified by Heywood et al (1979)
6 days/7 days =	Adjustment to account for exposure 6 days/week
1,000 =	Uncertainty factor. This uncertainty factor includes a factor of 10 to extrapolate from a LOAEL to a NOAEL, a factor of 10 to extrapolate from an animal species (dog) to humans, and a factor of 10 to account for potential sensitive human subpopulations.

5.1.2. Benchmark Dose Approach

The benchmark dose (BMD) approach utilizes mathematical models to characterize the dose-response curve for a given endpoint. Given a specified benchmark response (BMR) (e.g., a 10% increase in extra risk), the dose-response equation is used to calculate the BMD (the dose that yields the BMR), as well as the lower confidence limit of the BMD (referred to as the BMDL). The BMDL and/or the BMD are then used to derive the RfD (U.S. EPA, 1995).

5.1.2.1. Selection of Data Sets for Modeling

In accord with U.S. EPA guidance (U.S. EPA, 1995), several data sets in addition to the data set with the lowest LOAEL (Heywood et al., 1979) were selected for BMD modeling. This is because the study that identifies the lowest LOAEL may not always be suitable for modeling, or might not always yield the lowest BMD. The data sets selected for modeling included the following:

1) Incidence of fatty cysts in liver of dogs (Heywood et al., 1979)

2) Histological evidence of renal cytotoxicity in male rats exposed via drinking water (Hard et al., 2000)

3) Increased labeling index in kidney of female mice exposed via drinking water (Larson et al., 1994b)

4) Increased labeling index in liver of female rats exposed via gavage in corn oil (Larson et al., 1995b)

These studies were chosen because they all provide quantitative dose-response data for sensitive indicators of chloroform toxicity.

5.1.2.2. *BMD Modeling of Selected Data Sets*

The software employed for benchmark dose modeling was BMDS Version 1.2, downloaded from EPA's NCEA Web site.

Table 5 summarizes the five data sets that were used for BMD modeling. The data for dichotomous endpoints were fit to each of the dichotomous models provided in the software,

including gamma, logistic, multistage, probit, quantal-linear, quantal-quadratic, and Weibull. The BMR for dichotomous endpoints was a 10% increase in extra risk (U.S. EPA, 1995). Data for continuous endpoints were fit to each of the continuous models offered in the BMDS software (linear, polynomial, power, Hill), using a BMR of one standard deviation.

The detailed results of the BMD model fitting are presented in Appendix B. Within a data set, the preferred model was selected based on the quality of the model fit to the data. Models that yielded *p* values lower than 0.100 were judged to be inadequate and were not considered further. Models that yielded *p* values above 0.100 were assessed using Akaike's Information Criterion (AIC), along with a visual inspection of the quality of the fit (especially at low doses). The results are summarized in Table 6.

As seen, the kidney LI data set from Larson et al. (1994b) could not be adequately described by any of the continuous models. This is because even though the response was statistically significant, the magnitude of the response was small in comparison to normal variability, and the data did not form a smooth dose-response relationship (tending to first increase and then decrease as dose increased). The liver and kidney LI data sets from Larson et al. (1995b) were reasonably well fit by the Hill equation, with BMD values of 64-75 mg/kg/day. However, the software was not able to estimate a BMDL value in either case. The data sets from the studies by Hard et al. (2000) and by Heywood et al. (1979) were adequately fit by one or more of the dichotomous models, with the best fit being given by the log-logistic and the quantal-linear models, respectively. The preferred BMD of 70 mg/kg/day based on the renal cytotoxicity data of Hard et al. (2000) is similar to the BMD values derived for the LI data from Larson et al. (1995b), but is significantly higher than the preferred BMD based on the incidence of fatty cysts in dogs (1.7 mg/kg/day) reported by Heywood et al. (1979). The basis for this marked difference in BMD between studies is not known, but the data suggest that liver toxicity in the dog is a more sensitive endpoint of chloroform toxicity than renal or liver cytotoxicity in rodents.

5.1.2.3. *Calculation of the BMD-Based RfD*

Based on the calculations above, the BMDL value of 1.2 mg/kg/day derived from the study by Heywood et al. (1979) is selected as the most appropriate basis for the derivation of the RfD. Because this value is based on exposures that occurred 6 days per week, the value is adjusted as follows:

BMDL = (1.2 mg/kg/day) * (6/7) = 1.0 mg/kg/day

The RfD is derived from the BMDL by application of appropriate uncertainty factors (U.S. EPA, 1995). In this case, an uncertainty factor of 10 is used to account for interspecies extrapolation and a factor of 10 is used to protect potentially sensitive human subpopulations. Additional uncertainty factors are not required because the database for chloroform is complete. Bioassays are available in the dog (Heywood et al., 1979), and the rat and mouse (NCI, 1976; Jorgenson et al., 1982, 1985). Developmental toxicity studies are available in rats and rabbits exposed via the oral route (Thompson et al., 1974), and in rats (Baeder and Hoffmann, 1988,

Table 5. Dose-response data sets used for BMD modeling

Study	Endpoint	Dose	N	Mean	Stdev	Notes
Larson et al., 1994b	LI in kidney (medulla) of female mice exposed by drinking water for 3 weeks	0 15.7 42.7 82.5 184 329	14 14 14 14 14 14	2.3 2.2 3.8 3.5 2.7 3.2	0.7 0.8 0.2 0.5 1.5 0.6	Values are estimated from graph in Figure 6B of the report
Study	**Endpoint**	**Dose**	**N**	**Mean**	**Stdev**	**Notes**
Larson et al., 1995b	LI in kidney of female F344 rats exposed by gavage (3 wks)	0 34 100 200	10 10 10 10	1.3 1.5 22.4 33.8	1.0 0.3 20.9 20.9	High-dose group excluded from BMD modeling
Study	**Endpoint**	**Dose**	**N**	**Mean**	**Stdev**	**Notes**
Larson et al., 1995b	LI in liver of female F344 rats exposed by gavage (3 wks)	0 34 100 200 400	10 10 10 10 10	0.6 0.8 2.7 14.0 11.8	0.5 0.4 1.5 9.0 15.9	High-dose group excluded from BMD modeling
Study	**Endpoint**	**Dose**	**N**	**% Positive**		**Notes**
Hard et al., 2000	Renal proliferation in male OM rats (18 months)	0 19 38 81 160	18 16 19 19 17	0 0 0 58% 100%		Based on study by Jorgenson et al.
Study	**Endpoint**	**Dose**	**N**	**Incidence**		**Notes**
Heywood et al., 1979	Incidence of moderate to marked fatty cysts in liver of beagle dogs	0 15 30	27 15 15	1 9 13		Control group is based on vehicle control and does not include untreated animals

Table 6. Summary of noncancer BMD modeling results

Study	Endpoint	Range across models		Preferred model				
		P-Value	BMD	Type[a]	P-Value	AIC	BMD[b]	BMDL[b]
Larson et al., 1994b	LI in kidney (medulla) of female mice exposed by drinking water for 3 weeks	0.000-0.000	25.6-734	None	—	—	—	—
Larson et al., 1995b	LI in kidney of female F344 rats exposed by gavage (3 wks)	0.000-0.000	7.6-74.7	Hill	NA(c)	69.33	75	Failed
Larson et al., 1995b	LI in liver of female F344 rats exposed by gavage (3 wks)	0.000-0.007	15.7-63.6	Hill	NA(c)	158.55	64	Failed
Hard et al., 2000	Renal proliferation in male OM rats (18 months)	0.003-1.000	11.7-75.3	LL	1.000	27.86	70	46
Heywood et al., 1979	Incidence of moderate to marked fatty cysts in liver of beagle dogs	0.168-0.810	1.7-6.2	QL	0.810	44.58	1.7	1.2

[a]LL = log-logistic, QL = quantal linear.

[b]All BMD (benchmark dose) and BMDL (lower confidence limit on the BMD) values are reported to two significant figures.

[c]P value not calculated because degrees of freedom 0.

1991; Schwetz et al., 1974; Stanford Research Institute, 1978) and mice (Murray et al., 1979) exposed by the inhalation route. These studies indicate that effects on the fetus do not occur except at doses that cause maternal toxicity. A two-generation reproduction study (NTP, 1988) found no effects on fertility or reproduction at doses that resulted in liver histopathology. Finally, chloroform is rapidly metabolized and excreted and thus is not expected to bioaccumulate.

Based on all of these considerations, a total uncertainty factor of 100 is applied and the resulting RfD is 1E-02 mg/kg/day:

RfD = 1.0 mg/kg/day / 100 = 1E-02 mg/kg/day.

5.1.3. Summary of Oral RfD Derivation

In the derivation of the RfD for chloroform, both the traditional NOAEL/LOAEL and the benchmark dose approaches were used. In general, the NOAEL/LOAEL approach for derivation of an RfD is subject to a number of limitations, including the following (U.S. EPA, 1995):

- Identification of the NOAEL is often judgmental and sometimes controversial
- Experiments with low power tend to yield higher NOAELs
- The scope of the dose response curve plays little role in determining the NOAEL
- The NOAEL is restricted to doses tested

Most of these limitations are addressed by use of the BMD approach (U.S. EPA, 1995). Thus, the RfD based on the benchmark dose approach is generally preferred.

In the BMD analysis, we modeled several studies. The study by Heywood et al. (1979) was selected because it identified the lowest LOAEL and because it yielded the lowest BMD (more protective).

In this case, the dose-response data set from the critical study (Heywood et al., 1979) is composed of only two doses plus a control group. This is not ideal, because the shape of the dose-response curve is difficult to define with only three values, especially when the lowest dose yields a response that is well above the BMR. Nevertheless, the data do yield curve fits of adequate quality (see Appendix B), so the results of the BMD approach are considered to be preferable to the NOAEL/LOAEL approach.

Note that in this particular case, the two approaches (NOAEL/LOAEL, benchmark) yield equal RfD values. This is consistent, albeit coincidental, with the result from the default LOAEL/NOAEL approach.

5.2. INHALATION REFERENCE CONCENTRATION

Not available. The Agency is currently reviewing the literature and will develop a RfC at a later date.

5.3. ORAL CANCER ASSESSMENT

5.3.1. Choice of Approach

In accord with proposed EPA guidelines for cancer risk assessment (U.S. EPA, 1996a), the method used to characterize and quantify cancer risk from a chemical depends on what is known about the mode of action of carcinogenicity and the shape of the cancer dose-response curve for that chemical. A default assumption of linearity is appropriate when evidence supports a mode of action of gene mutation due to DNA reactivity or supports another mode of action that is anticipated to be linear. The linear approach is used as a matter of policy if the mode of action of carcinogenicity is not understood. A default assumption of nonlinearity is appropriate when there is no evidence for linearity and sufficient evidence to support an assumption of nonlinearity. Alternatively, the mode of action may theoretically have a threshold, e.g., the carcinogenicity may be a secondary effect of toxicity that is itself a threshold phenomenon (U.S. EPA, 1996a).

In the case of chloroform, the mode of action of carcinogenicity is reasonably well understood. Available data indicate that chloroform is not strongly mutagenic and that chloroform is not expected to produce rodent tumors via a genotoxic mode of action (ILSI, 1997). Rather, there is good evidence that carcinogenic responses observed in animals are associated with regenerative hyperplasia that occurs in response to cytolethality (ILSI, 1997; U.S. EPA, 1998c; U.S. EPA, 1998d). Because cytolethality occurs only at exposure levels above some critical dose, a nonlinear approach is considered to be the most appropriate method for characterizing the cancer risk from chloroform.

5.3.2. Quantification of Cancer Risk

The Proposed Guidelines for Carcinogenic Risk Assessment (U.S. EPA, 1996a) describe two alternative methods for quantifying the cancer risk from a chemical that acts via a nonlinear mode of action: a margin of exposure (MOE) evaluation based on quantitative modeling of tumor dose-response data, or use of an RfD approach. Each of these two alternatives were assessed as described below.

5.3.2.1. *Margin Of Exposure Analysis*

5.3.2.1.1. Choice of principal study. Table 7 summarizes studies that were considered candidates for the point-of-departure/margin-of-exposure quantification of the cancer risk from ingestion of chloroform. Chloroform has been reported to be carcinogenic in several chronic animal bioassays, with significant increases in the incidence of liver tumors in male and female mice and significant increases in the incidence of kidney tumors in male rats and mice (U.S. EPA, 1994d, 1998c). NAS (1987) reviewed all the available data on the carcinogenicity of chloroform and concluded that the quantitative risk estimate for oral exposure to chloroform should be based on the incidence of kidney tumors in male rats reported in the study by Jorgenson et al. (1985). This study is more appropriate than other candidate data sets in which exposure occurred via corn oil gavage, because exposure was via drinking water, the most applicable route of exposure for humans (U.S. EPA, 1998c). Use of data from drinking water

Table 7. Summary of inhalation noncancer studies in humans and animals

Effect category	Reference	Duration	Species/ strain	Gender	NOAEL			LOAEL			Basis
					ppm (raw)	mg/m³ (raw)	mg/m³ (TWA)	ppm (raw	mg/m³ (raw)	mg/m³ (TWA)	
Systemic effects	Phoon et al., 1983		1-6 mo.	Humans		—	—	—		70-2,000	Jaundice
	Li et al., 1993		1-15 yrs	Humans		—	—	—		13-30	Neurobehavioral effects; no effects on liver
	Challen et al., 1958		1-10 yrs	Humans		—	—	—		110-1,200	Subjective CNS effects; no clinical effects on liver
	Bomski et al., 1967		1-4 yrs	Humans		—	—	—		10-1,000	Enlarged liver, increased serum enzymes
	Templin et al., 1996a	13 wks (6 hr/d, 7 d/wk)	Rat: F344	M,F	—	—	—	2.0	9.7	2.4	Olfactory epithelial atrophy; effects on liver and kidney not apparent until 88 ppm (430 mg/m3)
	Templin et al., 1998	13 wks (5 d/wk, 6 hr/day)	Mouse: B6C3F1	M,F	5	24	4.3	23	112	20	Kidney histopathology in males (nasal effects not studied)
	Larson et al., 1996	13 wks (6 hr/d, 7 d/wk)	Mouse: B6C3F1	M,F	10	49	12	30	144	36	Incidence of liver effects in females and kidney effects in males
	Nagano et al., 1998	2 yr (6 hr/d, 7 d/wk)	Rat: F344 Mouse: BDF1	M,F M,F	— —	— —	— —	8.7 5	42 24	7.6 4.4	Necrosis and metaplasia of olfactory epithelium, ossification of turbinates
Reproductive/ developmental effects	Baeder and Hoffman, 1988	d. 7-16 of gest (7 hr/d)	Rat: Wistar	F	—	—	—	30	146	43	Maternal wt loss, embryolethality
	Baeder and Hoffman, 1991	d. 7-16 of gest (7 hr/d)	Rat: Wistar	F	3.1	15	4.4	10.7	52	15	Decreased weight in dams; fetal toxicity at 30 ppm
	Schwetz et al., 1974	d. 6-15 of gest. (7 hr/d)	Rat: Sprague-Dawley	F	30	146	43	95	464	135	Embryotoxicity, fetotoxicity
	Murray et al., 1979	d. 6-15 or 8-15 of gest (7 hr/d)	Mouse: CF1	F	—	—	—	100	488	142	Fetotoxicity, teratogenicity

TWA: time-weighted average.

studies eliminates a number of uncertainties regarding the potential impact of a corn oil vehicle, as well as reducing potential issues due to bolus vs. continuous low-dose exposure rate. The Jorgenson et al. (1985) data set is also preferred because of the multiple doses of chloroform used in the study (i.e., five doses including a control), and the large number of animals in the low-dose groups provides greater power in resolving the low end of the dose-response curve (U.S. EPA, 1998d).

5.3.2.1.2. Dose-response data. Jorgenson et al. (1985) administered chloroform in drinking water at concentrations of 0, 200, 400, 900, or 1,800 mg/L to male Osborne-Mendel rats for 104 weeks. Based on measured water intake and body weights, time-weighted average doses were 0, 19, 38, 81, or 160 mg/kg/day. The incidence of all kidney tumors combined (metastatic carcinomas, transitional cell carcinomas, tubular cell adenomas, and adenocarcinomas and nephroblastomas) in male rats increased in a dose-related manner. The incidences of all kidney tumors combined were 5/301 (2%), 1/50 (2%), 6/313 (2%), 7/148 (5%), 3/48 (6%), and 7/50 (14%) for the control, matched control, 200, 400, 900, and 1,800 mg/L groups, respectively. For the quantitative modeling performed here, the incidence of all kidney tumors in the matched control and the four treated groups was used.

5.3.2.1.3. Dose conversion. Because the exposure to rats in the Jorgenson et al. (1985) study was continuous in drinking water for the lifespan of the animals, no adjustment is needed to account for duration of exposure or duration of study. Doses in rats are converted to human equivalent doses by assuming that intakes (mg/day) are equivalent when scaled by body weight raised to the 3/4 power (U.S. EPA, 1992). When doses are expressed as mg/kg/day, this yields the following:

$$\text{Human dose} = \text{animal dose} \cdot \left(\frac{\text{BW}_{\text{rat}}}{\text{BW}_{\text{human}}} \right)^{1/4}$$

Based on an average body weight of rats in this study of 0.375 kg and an assumed human body weight of 70 kg, the human equivalent doses are 5.1, 10.3, 21.9, and 43.2 mg/kg/day.

5.3.2.1.4. Point of departure and margin of exposure. In accord with the proposed guidelines (U.S. EPA, 1996a), evaluation of the carcinogenic risk from a chemical that is believed to act via a nonlinear mechanism is done in two steps, as follows:

Step 1: Calculate the point of departure

The first step involves dose-response modeling of the cancer incidence data in the observable range to derive a point of departure (Pdp). The ED_{10} is defined as the dose that causes a 10% increase in the incidence of tumors, and the LED_{10} is defined as the lower 95% confidence limit on the dose associated with an estimated 10% increase in tumors. In general, the LED_{10} is taken as the point of departure.

Using the methods described in U.S. EPA (1995) and U.S. EPA (1996a), and employing EPA's BMDS Version 1.2 software, the dose-response data for male rat kidney were fit to a

variety of alternative mathematical models that are suitable for dichotomous responses. The results are presented in Appendix B and are summarized in Table 9. All of the models yielded an adequate fit with the data (all of the p values were greater than 0.50), and all yielded BMD values in the range of 34 to 38 mg/kg/day. This indicates that the estimates of the ED_{10} and the LED_{10} from this data set are not strongly model-dependent. In accord with EPA guidance, the preferred model was selected on the basis of the lowest AIC. This is the quantal-linear model, which yields an ED_{10} of 36 mg/kg/day and an LED_{10} of 23 mg/kg/day.

Step 2: Calculate a MOE

The MOE is calculated as the ratio of the Pdp divided by the measured or predicted environmental concentration. When evaluating the extent of the MOE from a risk management perspective, the assessor/manager can take into account guidance factors such as the following in determining whether the exposure is sufficiently protective of public health (U.S. EPA, 1998c,e):

1) A factor to account for potential intrahuman variability in susceptibility

2) A factor to account for the 10% effect level modeled by the $BMDL_{10}$ software for a cancer effect

3) A factor to account for remaining sources of uncertainty, including residual pharmacodynamic differences in sensitivity between the rat and human that may not be accounted for in the body-weight scaling; the relatively shallow slope of the dose-response curve; and using data from tumor rather than a precursor effect.

Other factors that could be considered in determining the MOE include the size of the population exposed, the duration and magnitude of human exposure, persistence in the environment and in the body, and the relative susceptibility of children and other potentially susceptible subpopulations. Humans are exposed to relatively low levels of chloroform (average 24 µg/L), below the level that is likely to induce a cytotoxic response. Further, there are no data to indicate that children are more susceptible than adults.

Comparing the point of departure (LED_{10}) of 23 mg/kg/day to the RfD of 0.01 mg/kg/day leads to a MOE of 2,000. The Agency considers this large MOE to indicate that the RfD is adequately protective of public health for cancer effects, based on the nonlinear dose response for chloroform and the mode of action for both cancer and noncancer effects having a common link through cytotoxicity.

Table 8. Summary of oral cancer studies in animals

Reference	Species	Sex	Vehicle	Duration	Doses (mg/kg/day)	N/grp	Observation
NCI, 1976	Rat: Osborne-	M, F	Corn oil	78 wks	90, 180	50	Increased incidence of kidney epithelial tumors in males
	Mouse: B6C3F1	M, F	Corn oil	93 wks	138, 237 (males) 238, 477	50	Increased incidence of hepatocellular carcinoma in males
Roe et al., 1979	Mouse: ICI, CBA, CF/1,	M, F	Toothpaste or oil	80 wks	17, 60	52	Increased incidence of kidney tumors in ICI males
Jorgenson et al., 1985	Rat: Osborne-	M, F	Water	104 wks	19, 38, 81, 160	53-330	Increased incidence of kidney tumors in male rats
	Mouse: B6C3F1	M, F	Water	104 wks	34, 65, 130, 263	50-430	No increase in tumors in female mice
Tumasonis et al., 1987	Rat: Wistar	M, F	Water	185 wks	150-200	32-45	Increased hepatic neoplastic nodules (females) and adenofibromas (males and
DeAngelo et al., 1995	Rat: F344	M	Water	100 wks	45, 90	50	Increased prevalence or multiplicity of hepatic hyperplastic or neoplastic lesions

Table 9. Dose-response modeling of male rat kidney tumor data

Ref: Jorgenson et al. 1985

Data Set: Male Kidney Tumors
Rat

Data Values:

Dose	HED	N	Incidence
0	0	50	1
19	5.1	313	6
38	10.3	148	7
81	21.9	48	3
160	43.2	50	7

Model	BMR	AIC	P value	BMD	BMDL	SETTINGS			
						Restrict Power > 1	Restrict Slope > 1	Restrict Betas > 0	N
Gamma	10% ER	195.33	0.637	34.5	22.8	yes			
Logistic	10% ER	193.94	0.673	37.9	29.9				
Log-Logistic	10% ER	195.33	0.639	34.4	22.5		yes		
Multi-Stage	10% ER	195.44	0.601	35.1	23.3			yes	2
Probit	10% ER	193.78	0.711	37.1	28.4				
Log-Probit	10% ER	194.41	0.546	36.1	25.4		yes		
Quantal-Linear	10% ER	193.64	0.744	36.2	23.0				
Quantal-Quadratic	10% ER	194.18	0.601	37.0	27.6				
Weibull	10% ER	195.34	0.633	34.6	22.9	yes			

Range Across Models

p-value	0.546	0.744
BMD	34.4	37.9

Preferred Model

AIC	193.64
Type	Quantal-Linear
p-Value	0.744
BMD	36.2
BMDL	23.0

5.3.2.2. *RfD Approach*

The Proposed Guidelines for Carcinogenic Risk Assessment (U.S. EPA, 1996a) state that when the mode-of-action analysis based on available data indicates that "the carcinogenic response is secondary to another toxicity that has a threshold, the margin-of-exposure analysis performed for toxicity is the same as is done for a noncancer endpoint, and an RfD for that toxicity may be considered in the cancer assessment."

This is the case for chloroform. That is, available evidence indicates that chloroform-induced carcinogenicity is secondary to cytotoxicity and regenerative hyperplasia, and that doses below the RfD do not result in cytolethality (and hence do not result in increased risk of cancer). Accordingly, the RfD developed above 1E-02 mg/kg/day for protection against noncancer effects (including cytolethality and regenerative hyperplasia) is also judged to be protective against increased risk of cancer.

5.3.2.3. *Summary of Oral Cancer Risk Evaluation*

As presented above, based on the mode of carcinogenic action for chloroform, a nonlinear approach is considered appropriate; two methods (MOE approach and the RfD method) were discussed for quantification of cancer risk from ingestion of chloroform. The RfD approach identified a dose of 0.01 mg/kg/day is not expected to produce either cancer or noncancer effects. Thus, this RfD is selected as protective against both noncancer and cancer effects of chloroform by the oral route.

5.4. INHALATION CANCER ASSESSMENT

[RESERVED]

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE-RESPONSE

6.1. HUMAN HAZARD POTENTIAL

6.1.1. Exposure Pathways

Chloroform is a volatile liquid that is sparingly soluble in water. It occurs in many public drinking water supplies as a byproduct of chlorination. Because chloroform is volatile, humans may be exposed to it in water not only by ingestion of drinking water, but also by inhalation of chloroform vapors released from water into indoor air. Dermal exposure may also occur during showering or bathing. Certain foods may contain chloroform. Chloroform may also be released to the environment from some chemical or industrial operations.

6.1.2. Toxicokinetics

Chloroform is readily absorbed following oral, dermal, and inhalation exposure. Once absorbed, chloroform is widely distributed throughout the body. Although chloroform may accumulate to some degree in body fat, most of an ingested or inhaled dose is readily excreted via the lungs, and there is little tendency for long-term bioaccumulation in the body.

Chloroform is metabolized mainly in the liver, but metabolism also occurs in other tissues such as the kidney. Metabolism may occur via two pathways, oxidative and reductive. Both pathways occur in the cytoplasm and are mediated by cytochrome P450. Oxidative metabolism results in the conversion of chloroform to phosgene and hydrochloric acid, both of which are toxic to cells. Reductive metabolism results in formation of dichloromethyl free radical, which is highly reactive and can cause lipid peroxidation. Under normal conditions, oxidative metabolism is thought to be the primary metabolic pathway, and reductive metabolism is believed to be relatively minor.

6.1.3. Characterization of Noncancer Effects

Data on the noncancer effects of chloroform in humans are limited. Central nervous system effects have been reported in workers exposed to high levels of chloroform in workplace air as well as in individuals ingesting high doses. However, central nervous system effects are not likely to be of concern following low level inhalation or oral exposures. Humans exposed to chloroform by either inhalation or oral routes often display signs of liver damage (Phoon et al., 1983; Bomski et al., 1967; Schroeder, 1965), supporting the conclusion that the adverse effects in humans are similar to those observed in animals.

Chloroform toxicity has been extensively studied in animals. In general, similar patterns of adverse effects are observed following both oral and inhalation exposure. Effects occur mainly in liver, kidney (epithelial cells of the proximal tubule), and the olfactory nasal epithelium. It should be noted that the nasal effects are a result of absorbed chloroform rather than an effect of direct contact, as nasal lesions occur following both oral and inhalation exposure (Larson et al., 1995b; Templin et al., 1996a), and the spatial pattern of lesions in the nasal passage does not correlate with the airflow-driven pattern of chloroform contact with epithelial surfaces (Mery et al., 1994).

Effects on liver, kidney, and nasal epithelium are generally characterized by cellular degeneration, vacuolization, and necrosis, often accompanied by changes in organ weights and/or organ function. In some cases, evidence of cellular regeneration (presumably in response to antecedent cellular necrosis) can be detected even when frank cytotoxicity is not readily apparent. Effects on liver are largely reversible, whereas effects in the kidney and the nasal epithelium are poorly reversible.

The severity of liver injury following oral exposure depends in part on the dosing vehicle. For example, effects that occur following exposure in water are generally less than following an equal dose administered in corn oil. The basis for this vehicle effect is not certain, but may be

due to toxicokinetic differences between bolus versus continuous exposure, with bolus exposures resulting in higher concentrations delivered to cells than with drinking water exposures.

6.1.4. Reproductive Effects and Risks to Children

Exposure of animals to chloroform during pregnancy can also result in reproductive or developmental toxicity. However, available studies indicate that these effects occur only at the same or higher doses as those which cause effects on the dam (U.S. EPA, 1998d), suggesting that most of the effects are secondary to maternal toxicity. No studies were located that demonstrate that the fetus is more sensitive to chloroform toxicity than the mother. This is supported by findings that the enzyme responsible for chloroform metabolism (CYP2E1) is low or absent in the fetus (Hakkola et al., 1998).

6.1.5. Mode of Toxicity

The toxicity of chloroform on liver, kidney, and nasal mucosa is clearly related to the ability of tissues to metabolize chloroform. This is based on the finding that toxicity occurs in those tissues that have the greatest ability to metabolize chloroform, and that toxicity can be increased or decreased by agents that increase or decrease the activity of the metabolic enzymes, respectively. Similarly, there are clear differences between genders, strains, and species in their relative sensitivity to chloroform, and these differences in toxicity correlate with differences in metabolic capacity. For example, renal toxicity is usually more severe in males than females, apparently because the cytochrome P450 chiefly responsible for the metabolism of chloroform (CTP2E1) is induced by testosterone. No data were located on the mode of action or the role of metabolism in the neurological effects of chloroform.

The molecular mechanism by which chloroform metabolism results in cellular toxicity is not certain, but is probably due mainly to the formation of phosgene as a result of oxidative metabolism. Phosgene is highly reactive and can bind with and inactivate cellular molecules. This mode of toxicity is supported by the finding that glutathione protects against the toxic effects of chloroform and that toxicity becomes manifest only after glutathione levels have been depleted (Brown et al., 1974; Stevens and Anders, 1981; Docks and Krishna, 1976; ILSI, 1997). Oxidative metabolism of chloroform also produces hydrochloric acid, which may contribute to the toxic effect.

6.1.6. Characterization of Human Carcinogenic Potential

There are no data from studies in humans that are adequate to directly assess the potential carcinogenicity of chloroform to humans.

In animals, chloroform has been shown to cause increased incidence of liver and kidney tumors in several species by several exposure routes. However, this carcinogenic response occurs only at high dose levels that result in cytotoxicity, and the weight of evidence indicates that carcinogenic responses observed in animals are associated with regenerative hyperplasia that occurs in response to cytolethality (U.S. EPA, 1998c; U.S. EPA, 1998d). In mouse liver, numerous studies have demonstrated the association of tumor and cytotoxicity (e.g., Larson et al.,

1994b,c, 1995a). In mouse kidney, ILSI (1997) reviewed the available data on cytolethality and carcinogenicity and concluded that in the proximal tubule in the male mouse kidney there was a strong association between the occurrence of chloroform-induced cytotoxicity and regenerative hyperplasia, and the development of tumors. More recently, Hard et al. (2000) did a histological reevaluation of the original kidney tissue preparations from the various time points of the 2-year chloroform drinking water bioassay in male rats and established the presence of renal tubule cell alterations consistent with chronically persistent cytotoxicity and cell turnover at dose levels associated with renal tubule tumor induction. Such changes were confirmed also in the NCI oral gavage bioassay. Based on the correlation, ILSI (1997) concluded that the data indicate that phosgene and other metabolites formed via the oxidative route of metabolism are responsible for the cytotoxic effects and subsequent cell proliferation and development of tumors in the kidney following exposures to chloroform. Because the carcinogenicity of chloroform is secondary to cytotoxicity which has a threshold, and because chloroform and its metabolites are not strongly genotoxic and are unlikely to cause cancer by a genotoxic mechanism (Lohman et al., 1992; ILSI, 1997; Golden et al., 1997; WHO, 1998), a nonlinear approach is more appropriate for low-dose extrapolation.

Under the Proposed Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1996a, U.S. EPA, 1999), chloroform is *likely to be carcinogenic to humans by all routes of exposure* under high-dose conditions that lead to cytotoxicity and regenerative hyperplasia in susceptible tissues (U.S. EPA, 1998c,d). Chloroform is *not likely to be carcinogenic to humans by any routes of exposure* at a dose level that does not cause cytotoxicity and cell regeneration. This weight-of-evidence conclusion is based on observations that chloroform is absorbed by all routes of exposure, and studies in animals exposed by both oral and inhalation pathways, which indicate that sustained or repeated cytotoxicity with secondary regenerative hyperplasia precedes, and is probably a causal factor for, hepatic and renal neoplasia. This conclusion is supported by the finding that chloroform is not a strong mutagen and is not likely to cause cancer through a genotoxic mode of action. There are no epidemiological data specific to chloroform and, at most, equivocal epidemiological data related to drinking water exposures that cannot necessarily be attributed to chloroform amongst multiple other disinfection byproducts.

6.2. DOSE-RESPONSE

Data from studies of chloroform toxicity in humans are not sufficient to support derivation of reliable quantitative risk estimates. Therefore, quantitative estimates of risk as a result of low-level chronic exposure to chloroform are based on animal experiments.

6.2.1. Oral RfD

The most reliable study for the derivation of the chronic oral RfD is the report by Heywood et al. (1979). In this study, dogs were exposed to chloroform in toothpaste base for 7.5 years. Chloroform doses of 15 mg/kg/day resulted in an increase in the severity of fatty cysts in liver. Dose-response data from this study were evaluated using the BMD approach, and the most reliable estimate of the lower bound on the benchmark dose ($BMDL_{10}$) was 1.0 mg/kg/day. The RfD was derived from this value by application of an uncertainty factor of 100 to account for

extrapolation from animals to humans and for potential variability in sensitivity between humans. The resulting RfD is 0.01 mg/kg/day.

The overall confidence in the RfD is medium. The RfD is based on the increase in severity of "fatty cysts" in the livers of dogs exposed for 7.5 years to chloroform in a toothpaste base. Although fatty cysts are not a common endpoint, fat accumulation in the liver is a common and characteristic effect of chloroform exposure (see Roe et al., 1979; Jorgenson and Rushbrook, 1980; Jorgenson et al., 1982; DeAngelo et al., 1995; Thompson et al., 1974), supporting the view that fatty cysts are an authentic and toxicologically relevant endpoint. One important aspect of the study is that these fatty cysts occurred at doses much lower than those that were required to produce fat accumulation or cytotoxicity in rodents, suggesting either that the dog is a more sensitive species than rodents or that the long-term exposure period allowed the detection of effects that were not observed in shorter term studies. Because chloroform was administered in a toothpaste base that contained glycerol and small quantities of natural oils, it is plausible that these constituents may have potentiated the effects of the chloroform. However, this is not judged to be a likely explanation for the effect. On the basis of these considerations, confidence in the principal study is rated as medium. The confidence in the remainder of the database on chloroform toxicity is medium. Chloroform has been evaluated in a number of chronic and reproductive/developmental studies, and data are sufficient to conclude that effects on liver are the critical (most sensitive) effect.

6.2.2. Inhalation RfC

[RESERVED]

6.2.3. Oral Cancer Risk

In accord with the Proposed Guidelines for Carcinogenic Risk Assessment (U.S. EPA, 1996a), when available evidence indicates that a carcinogenic response is secondary to another toxicity that has a threshold, the MOE analysis performed for toxicity may be the same as is done for a noncancer endpoint. This is the case for chloroform. That is, available evidence indicates that chloroform-induced carcinogenicity is secondary to cytotoxicity and regenerative hyperplasia, and that doses below the RfD do not result in cytolethality (and hence are unlikely to result in increased risk of cancer). Accordingly, the RfD developed above (1E-02 mg/kg/day) for protection against noncancer effects (including cytolethality and regenerative hyperplasia) can also be considered protective against increased risk of cancer.

6.2.4. Inhalation Cancer Risk

[RESERVED]

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APPENDIX A. EXTERNAL PEER REVIEW— SUMMARY OF COMMENTS AND DISPOSITION

The Toxicological Review of Chloroform was derived from "Health Risk Assessment/Characterization of the Drinking Water Disinfectant Byproduct Chloroform" and the Mode of Action Analysis for the Carcinogenicity of Chloroform (SAB review draft). Both documents have gone through internal and external reviews.

External Peer Reviewers and Affiliations

James A. Swenberg, D.V.M., Ph.D., University of North Carolina
Lorenz Rhomberg, Ph.D., Gradient Corporation
Sandra Baird, The Baird Group
R. Julian Preston, Ph.D., Chemical Industry Institute of Toxicology

External peer reviewers' comments and the disposition of their recommendations are as follows.

Summary & Response of External Reviewer Comments

Health Risk Assessment/Characterization of the Drinking Water Disinfection Byproduct Chloroform, March 13, 1998

Comments below are either actual text or paraphrases from the reviewers' comments. Editorial revisions and specific suggestions were invariably made in response to comments by the reviewers. These comments and responses are not reviewed here.

James A. Swenberg, D.V.M., Ph.D.
University of North Carolina

Section, Comment, and Response

2.1.1. Toxicokinetics

The summary should state more definitively that the oxidative pathway is responsible for most metabolism. Excretion of chloroform by the lungs is dose dependent, with greater amounts excreted unchanged at high doses.

Response: Summary was changed as suggested.

2.1.4. Mechanisms of Toxicity

The section on formation of DNA adducts needs revision. DNA adducts have not been identified with chloroform. Very small amounts of "covalent binding" have been reported.

These are not the same. They may only represent metabolic incorporation from the C-1 pool. The reductive pathway is associated with free radical formation, which leads to oxidative stress and lipid peroxidation. Such responses are highly nonlinear and are enhanced by conditions that deplete cellular defenses such as glutathione. Under normal conditions, a cell is well equipped to detoxify these free radicals. The Melnick paper further confuses the issue by including brominated compounds that are generally recognized as genotoxic and that have identifiable DNA adducts.

Response: Several clarifying changes were made in the text on these issues.

2.2.2. Quantification of Carcinogenic Effects

This section is rather confusing compared to the ILSI document. I do not understand the factor of 3 for slope, nor the factor of 10 for severity. The cancer endpoints are associated with toxicity and are expected results for the cause and effect. If cytotoxicity is used instead of tumors, a factor of 10 and the use of the ED10 may be reasonable. This can be coupled with a factor of 10 for intrahuman variability and a factor of 3 to 10 for interspecies variation, providing a margin of exposure (MOE) between 300 and 1,000. The ILSI document uses a factor of 30. This should be discussed. This section of the document needs to be more transparent and its content presented as a range throughout the document.

Response: Text was changed to add clarity but the range of MOE was not specified. Rather, a single value of 1,000-fold was chosen.

3.1.2. Strengths and Weaknesses

This section is very brief. In comparison with the ILSI document, it is much less informative and less transparent. There is no discussion of inconsistencies in cell proliferation data and strain-specific deficiencies in the data sets.

Response: The text was changed only slightly here. Reference is made to the ILSI work. This text could be greatly expanded but such effort would increase the overall length beyond that desired for this risk characterization text.

3.1.3. Key Conclusions, Assumptions, and Defaults

Again, this section is very brief and less informative than the ILSI document. The last part of paragraph 1 is incorrect in calling the DNA damage that results from reductive metabolism "direct." It is associated with free radicals and is fully expected to be nonlinear and highly correlated with depletion of cellular defense mechanisms.

Response: Changes were made to correct the first paragraph. This section was combined with the weight of evidence section.

3.1.4. Weight of Evidence

Once again, the document should not refer to the free radical mechanism for DNA damage as "direct" and it should not be stated to be linear as a function of dose. I know of no data that demonstrate this, but there are many data sets that speak against it.

Response: Changes were made to correct this section. This section was combined with that described above.

3.1.7. Alternative Conclusions

This may lead to high-dose genotoxicity from free radicals that could contribute to chloroform's carcinogenicity. This pathway is recognized to be a minor one that would not be expected to contribute to low-dose carcinogenicity.

Response: Text was changed to reflect the concept of minor pathway.

3.2.1. Overall Conclusions

This section automatically embraces 7 µg/kg-day as the MOE-based dose. This uses a factor of 3,000. In my opinion, this should be given as a range using a MOE. ILSI would go as low as 30. Thus, this section should be broadened to better reflect a range of MOE.

Response: The range of a MOE was not specified, but an increase in transparency was attempted. An overall MOE of 1,000 was chosen as best representative of the underlying data.

3.2.2. Human Susceptibility

This section addresses only differences in exposure. It also needs to address genetic polymorphisms that change metabolism. The interhuman factor of 10 has been used for MOE and should be put in context with this section.

Response: Section was changed as suggested.

3.3.1. Overall Conclusions

Again, this section uses only the MOE of 3,000. The range needs to be more inclusive.

Response: Again, the range of a MOE was not specified, but an increase in transparency was attempted. An overall MOE of 1,000 was chosen as best representative of the underlying data.

3.3.7. Significant Issues and Uncertainties

As stated several times in this review, tumors would not be expected "to be evoked in a linear fashion by gene mutations from free radicals via reductive metabolism." Such mechanisms are expected to be nonlinear and associated with high doses that deplete cellular defense mechanisms.

The MOE should be discussed as a range, not as 3,000. This number is highly conservative and not well supported by the data presented.

Response: The section was revised to address the comments on the expected lack of low-dose linearity with tumors. Again, the range of a MOE was not specified, but an increase in transparency was attempted. An overall MOE of 1,000 was chosen as best representative of the underlying data.

3.3.8. Alternative Approaches

Again, the free radical mechanism is not expected to be linear.

Response: Section was revised to reflect this comment.

Lorenz Rhomberg, Ph.D.
Newton, MA

Comment:

Critical discussion of key matters not included in the risk characterization text, such as chloroform's metabolism, the role of corn oil vehicle, the agent's genotoxic potential, and the central hypothesis that animal tumors are secondary to cytotoxicity and compensatory cell proliferation in the target tissues. In sum, the risk characterization does not really document the Agency's weighing of the evidence on these key questions.

Response: The commentor is correct in that the text does not go into detail regarding key matters. However, this text is intended to be a characterization of the risk, geared more to risk managers. Previous manuscripts, appropriately referenced in this text, are available for a fuller explication of the key matters that were only summarized here.

Comment:

The risk characterization document contains no data. Although it need not be comprehensive, it would seem important to present (in tabular form) the key data sets upon which the quantitative risk assessment calculations are based, as well as other data sets that stand as the most prominent alternatives. The administered doses and tumor counts of the key Jorgenson male rat kidney tumor data are reported differently in Chiu et al. (1996) and in the ILSI document, for instance, and it is important that the specific version used in the final calculations (and the reasons for its variation from other versions) be documented.

Response: Tumor counts were added for the key studies. The chosen data set can be viewed on EPA’s IRIS site (www.epa.gov/iris), and was appropriately cited.

Comment:

Similarly, there is no documentation of risk assessment calculations, either in dose metric calculation or in dose-response analysis. The citation for the key dose-response analysis (p. 19) to "personal correspondence" with Paul Pinsky of EPA's National Center for Environmental Assessment is not adequate documentation for so central a calculation. It makes it impossible for the reader (and for me as a reviewer) to verify the calculations or to see what specific approaches were taken (for instance, regarding such matters as lower bound determination, allowance for concurrent mortality, and degree of model).

Response: This document is the original source of the data set used for the cancer analysis. Citation of a personal communication with the EPA scientist that did this analysis is appropriate.

Comment:
Elsewhere (p. 34) it is implied that the cross-species equivalency of doses was assumed to be based on surface area scaling (i.e., $mg/kg^{2/3}$/day equivalency), although the calculations are said to follow the draft Proposed Guidelines, which mandate $mg/kg^{3/4}$/day as an oral dosing default. The document should show key calculations so that such matters can be checked and the calculation's basis understood.

> **Response:** The commentor is correct. The 3/4 power of body weight was used in the assessment and is appropriately acknowledged in a footnote to the text.

Comment:
Even though reviews of the literature and the state of the science on areas such as epidemiology, animal bioassays, metabolism and pharmacokinetics, and mechanisms of toxicity need not be included in the risk characterization per se, a succinct summary of the main issues (as opposed to the main studies and their findings) would seem to be in order. In the case of chloroform, for epidemiology the main issues are attribution of water chlorination effects to specific disinfection byproducts and the confounding of exposure to chlorine-disinfected drinking water with other factors of potential importance to cancer risks; for animal bioassays they are the inconsistencies among studies, the dependence on route of administration, the assessment of cytotoxicity among bioassay subjects, and the potential bearing of minor tumor responses other than those in kidney and liver; for metabolism the issue is the extent of reductive metabolism in vivo; for pharmacokinetics the issues are the incomplete understanding of the role of corn oil, the characterization of metabolism in humans, and the question of the most appropriate internal dose measure for understanding the relation of tissue toxic effects as a function of dosing regime; for toxicity mechanisms the issues are the possibility of significant genotoxicity in the target organs for carcinogenicity, the mechanistic role, if any, of reductive metabolism, the correlation of cytotoxicity and tumors in the bioassays, the dose-response patterns for cytotoxicity, the nature of the evidence that cytotoxicity is necessary for chloroform's tumorigenesis (and not just ancillary), and the link of quantitative measures of cell proliferation to quantitative changes in cancer risk.

> **Response:** Several of these issues were further discussed as suggested by the commentor, but others were given only cursory treatment, owing in part to the overall limitations of this text as a risk management tool.

Comment:
The current draft risk characterization refers to some of these issues (although not all of them), but it tends to do so simply by citing the existence of the arguments. As a result, when it comes time to make choices of analytical approaches, the reader is left without a strong sense of why the Agency is choosing the path it takes or how much confidence it invests in its chosen approach. For example, the key section on Mechanism of Toxicity (pp. 13-17) mentions various relevant studies and notes their differing implications, but it takes no stand on the issue of whether chloroform's observed animal carcinogenicity should be considered secondary to (and causally dependent on) cytotoxicity in the target organs. Yet immediately afterward, and without any discussion, the document states (p. 19), “In view of the weight of evidence that chloroform may induce tumors by a nonlinear mechanism (ILSI, 1997), a margin of exposure approach for dose response analysis [sic]." (One can easily imagine this incomplete sentence having arisen from a

hesitancy between finishing it with "is chosen" versus "is considered," with neither being chosen in the end.)

Response: Attempts were made to clarify the Agency's position throughout this text.

Comment:
In my view, something much bolder is needed, even if the outcome is to boldly declare one's uncertainty. As I argue below, I think the Agency would err if it put all its confidence in the existing "nonlinear" dose-response method, but if it is to do so, it should more explicitly declare its reasoning for why such a course is appropriate. After all, it reverses the earlier Agency stance. As it stands, the conclusions are simply (albeit hesitantly) declared without clearly stated reasons.

Response: Statements were made more boldly throughout the text that the nonlinear approach to the assessment of chloroform's carcinogenic risk at low doses was consistent with more of the data than were alternative hypotheses.

Comment:
A better discussion of corn oil gavage is warranted because the differences in cytotoxicity and in tumorigenesis between drinking water and oil gavage experiments are critical to choosing the Jorgenson study and to arguments about the most appropriate internal dosimeter. On p. 6 it is noted that chloroform is actually absorbed more readily from aqueous vehicles, and the reference to the role of first-pass metabolism seems inappropriate because it applies equally to all oral exposures. To me, the magnitude of the corn oil effect seems puzzling, and confidence in other mechanistic explanations of cytotoxicity hinges on the strength of the dose-rate explanation. This needs to be noted as an issue.

Response: EPA (1987) has discussed this issue and decided to use the Jorgenson study. This text merely confirms the previous decision. This discussion is highlighted in EPA's IRIS (EPA, 1998).

Comment:
Even for drinking water contamination, exposure by inhalation can be a major route of uptake (as noted briefly in the document's exposure section). Yet elsewhere the issue of inhalation is not mentioned, despite the existence of the positive Japanese inhalation study. Is the earlier EPA inhalation unit risk for chloroform obviated, or does it stand in the absence of an explicit revision? How are inhalation exposures, and combined inhalation and oral exposures, to be assessed dosimetrically?

Response: The inhalation study is not yet published, but these issues should again be considered after it is.

Comment:
There are several questions about metabolism that bear further discussion. No clear statement is given regarding the weight to be given to the possibility of reductive metabolism in vivo to dichloromethyl radicals. What is the evidence that this occurs in vivo? If the free radicals are thought to be potentially genotoxic, where is the evidence of such genotoxicity? (After all, the

genotoxicity tests are not specific to damage caused by oxidative metabolism; the only question is whether metabolic patterns typical of the in vivo situation are examined. Why is there no unscheduled DNA synthesis in the liver when animals are exposed in vivo, for instance?)

The impact of having two P450 isozymes with activity toward chloroform is not examined. If CYP2B 1/2 are active at high doses, what does this say about relative high- and low-dose metabolic activation?

> **Response:** The section on metabolism was revised to further discuss the weight of evidence for the reductive metabolic pathway. Also, text was added to further discuss the P450 metabolism.

Comment:
The document tends to equate reductive metabolism with genotoxicity (and hence linear dose-response approaches) and oxidative metabolism with cytotoxicity (and nonlinear approaches). It implies that, if one doubts the operation of reductive metabolism, nonlinear approaches are indicated. This seems inappropriate for several reasons. First, as noted, the in vivo genotoxicity data do not refer to which reactive moiety may be causing effects, and the lack of effects speaks equally to the genotoxic potential of metabolically generated free radicals and metabolically generated phosgene. Second, free radicals attack a variety of cellular macromolecules, notably lipids, and they may serve as agents of cytotoxicity as well as phosgene. This effect may, however, have different sensitivity to the *rate* of metabolism, an argument that plays importantly in the choice of internal dosimeters. Third, genotoxicity is not the only reason to rely on a linear approach to dose-response analysis, and lack of genotoxicity should not be deemed a sufficient reason to undertake only an MOE approach.

> **Response:** Text changes were made throughout the document in order to break the unduly rigorous connection between reductive metabolism and linearity, and oxidative metabolism and nonlinearity.

Comment:
As noted above, the key studies should have the data presented in tabular form. It should be clear how issues such as concurrent mortality are handled. In particular, the Jorgenson study has some issues with early mortality.

> **Response:** The data from the cancer bioassays were presented in the test. This text relied on EPA's IRIS file for current interpretation of these bioassays.

Comment:
At one point, EPA was making much of tumor responses in the Jorgenson study other than the kidney tumors in rats (e.g., possible elevation of mouse lymphomas). The current assessment presumes that only mouse liver and rat kidney tumors are at issue. A clear statement of the basis for choosing which tumor responses warrant investigation is in order.

> **Response:** These issues have been discussed previously in EPA texts. No need existed to repeat the earlier arguments.

Comment:
Characterization should have a concise summary (probably tabular) of the results on target organ doses (examining the contending dose metrics) in different species and organs following chloroform administration by different routes, as implied by the pharmacokinetic modeling. This will aid in examining the question of how species differences in response are explained by dosimetric differences.

> **Response:** PBPK modeling was not used in this text, other than to reference the ILSI report. Thus, a table of these values was not considered necessary. Furthermore, this text is a risk characterization document, geared more for review by risk managers. Technical details are appropriately referenced in other previous work.

Comment:
Similarly, a table should summarize the results of target organ cytotoxicity in the animal bioassays to aid in examining the question of the correlation of cytotoxicity and tumorigenicity. Also, graphs of the results of the series of CIIT studies on labeling index at different exposures (similar to those shown in Chiu et al., 1996) should be shown. These should be x-y graphs, not bar graphs, so that the shape of the labeling index response curve with exposure is evident.

> **Response:** These data are interesting and cited as part of the ILSI report. However, they are not used here and would only add text to an already "full" risk characterization document.

Comment:
The equation of genotoxicity with the reductive pathway evident in the document is misguided in my view, as argued earlier.

> **Response:** Text was changed to reflect this concern.

Comment:
From the foregoing, I imagine it is evident that I feel that the "nonlinear" approach as set out in the Proposed Guidelines is too readily invoked and that it poorly accommodates what we know and do not know about the carcinogenic process, even when that process is thought to be secondary to a nonlinear biological precursor effect such as cytotoxicity. In criticizing the application of this approach to chloroform, I am not invoking genotoxicity nor even denying cytotoxicity as the key mode of action.

The rationale behind the nonlinear method is that at some moderately low doses the dose-response curve for tumors becomes sufficiently steep in its decline with exposure level that smaller exposures will be without meaningful risk. If human exposures are well below those associated with tumors in animals, it is supposed that one can be assured that the intervening steep drop is sufficient to render the low exposures safe. The unanswered question is how far below is "well below," and what evidence can be adduced to judge the adequacy of the size of the margin-of-exposure?

Logically, the approach should be to examine the dose-response pattern for the biological effect to which carcinogenesis is deemed secondary, and which is presumed to have an actual or practical threshold. In practice, the method assumes that this point of steep decline to trivial risk levels will be found somewhere slightly below the ED10 for tumors.

The risk characterization document accepts this presumption quite readily; it calculates a point of departure as the ED10 (or LED10) for rat kidney tumors from the Jorgenson drinking water study, and then concentrates its discussion to the size of uncertainty factors that might be applied. Actual dose-response patterns are not examined, but they are presumed to be sufficiently nonlinear. Also, the metabolic activation of chloroform is stated as "nonlinear as a function of dose" (p. 28). A plot of these data does not necessarily lead to this conclusion.

In sum, these data seem to provide a poor basis for invoking a method that presumes that risk is highly nonlinear just below the ED10 for tumors. This shows that a good deal more effort needs to be put into defining an appropriate point of departure, and that the adequacy of the point chosen needs to be examined and defended in a way that has not yet been done.

> **Response:** A number of interesting and important issues are raised in this comment that apply to chloroform, but perhaps more importantly, to the ongoing revisions of the cancer guidelines of EPA. Specific text changes were made in the chloroform text to downplay the nonlinearity with dose, especially in the area of metabolism. However, the recommendation to use an MOE approach rather than a linear extrapolation is still retained with chloroform, because, of the two available methods, this seems to best fit the available data. This judgment is consistent with EPA's 1996 guidelines.

Comment:
I question whether the uncertainty factor approach is appropriate at all. Nonetheless, one can examine the document's reasoning for the size of the various factors.

Three-fold factor for slope

Shallower slopes warrant larger margins, but how big should such a margin be? Allowing threefold below an ED10 for slope (p. 19) with a probit slope of 0.62 leads to a dose that has a projected risk of 2.5%. (A 10-fold factor only goes down to 0.3%, as opposed to the linear method's 1.0%). Because this is the only factor that is designed to address the nonlinearity and drop to acceptable doses, it would seem that in the present case a factor of 100 might be just marginally enough (i.e., going down to a 10^{-4} risk on the fitted nonlinear probit curve).

The point of the nonlinear method is to avoid extrapolating a fitted curve down to low-dose. But threefold below the ED10 is still within the observed range (it is about at the level of the second dose group in the Jorgenson study) and is in the range where increasing tumor risk is empirically evident.

Tenfold factor for severity of endpoint

I do not understand the logic of this factor. It has not been the usual practice to assume site concordance and to presume that the potential human endpoint will be for the same tumor in terms of histological type and malignant aggressiveness. If the argument is that the rat tumors were frequently benign, is it the presumption that a factor needs to be included in case the human tumors are less so? If the rat tumors were all malignant, would this factor then be smaller (on the premise that human tumors could only be less malignant, not more)? I also do not understand why the "closeness" of the cytotoxicity and tumorigenicity curves (an odd concept in itself, as one is continuous and the other quantal) leads to a lesser factor.

Tenfold factor intrahuman variability

This presumably means among-human variability (rather than intraindividual variability). When assessing population risk, one should presumably not use this factor, because it allows for vulnerability in individual risk in some people, but in a population high individual risks on the part of some will be balanced by lower than average risks in others. The relative CYP2E 1 activity is a poor basis for judging such variability, because it is not established that metabolic activation is proportional to this activity (although the PBPK models could examine this question).

Tenfold factor persistence

Because the animal studies are for lifetime exposure, it is not clear what a correction for persistence is for. Presumably, this is covered by the dosimetry assumptions about equivalently toxic dose rates in humans and animals.

Tenfold factor interspecies variation

This has the same ambiguity that the analogous factor has in noncancer risk assessment. Is the factor an adjustment for an expected sensitivity difference (as implied by the discussion of the surface area adjustment to doses as obviating it), or is it an allowance for the possibility that for this particular agent, humans are particularly susceptible owing to some cause not covered by the dosimetry or other adjustments that are made? I note again that the 2/3-power adjustment mentioned is at odds with the methodology specified in the Guidelines Proposal. The reference to assumed kinetic differences is inappropriate, as the factor is applied to account for general differences in the pace of physiological processes, including those that control the pharmacodynamic response.

The result of applying these factors is that the "acceptable" dose is only 40-fold higher than the dose producing a 10^{-6} risk under the linear method. Given that the allowance for slope is clearly too small, this raises the question of whether the nonlinear method is meaningfully different from the linear method. It differs mostly in relying on ill-defined arguments for how large the margin-of-exposure must be in order to be deemed safe.

> **Response:** A number of interesting and important issues are also raised in this comment that apply to chloroform, but perhaps more importantly, to the ongoing revisions of the cancer guidelines of EPA. In part based on this comment, specific text changes were

made in the chloroform text to downplay the numerous discussions of factors with various pieces of the data. The end result was the choice of a 1,000-fold MOE. This choice is consistent with the overall amount of available data for chloroform, but also accounts for potential sensitive populations. The use of MOE is new in EPA dose-response assessment for carcinogens. Improvements to the process are likely to occur as additional chemicals are considered for this procedure.

Comment:
It seems unfortunate not to explore the implications of internal dose measures more thoroughly. A lot has been done in this arena, and an interesting analysis was conducted in the ILSI Panel document. In that case, I had objections to the use of the ED10 as a point of departure for similar reasons that I raise above. Nonetheless, the dose-response analysis shows consistency among different data sets when internal doses are used, allowing more confidence in the description of the relationship in the observed range. I refer to my comments on the ILSI document on this matter.

Response: This risk characterization text was intended to synthesize previous work into a coherent picture for risk managers, and not to enhance the technical work of the ILSI or other previous EPA deliberations. Although our understanding of the chloroform database still can be improved, sufficient data are available to develop a nonlinear approach to the estimation of low-dose cancer risk for chloroform within EPA's new cancer guidelines.

Comment:
The case of chloroform is complex, difficult, and challenging. It is a formidable task to bring rigorous discussion of the many issues into a single, readable, useful risk characterization. The authors of the risk characterization have made a good try, but they have been hampered by the lack of defined stances on the issues and clearly articulated bases for analytical approaches among the source material. The risk characterization is supposed to summarize and communicate these judgments, not to make them de novo. I am sympathetic with the challenge faced by the authors, but I am compelled to review the document according to what ideally could be accomplished, and what ideally should be covered in a fully developed risk assessment for a compound with a database as rich as that of chloroform.

Many of my comments on the nonlinear method and the margin-of-exposure stem from doubts about the method as set out in the Proposed Guidelines, especially regarding the wisdom of using an ED10 as a point of departure.

I do not disagree markedly with the basic conclusions of the risk characterization regarding the likely levels of human risk from exposures as actually experienced, although I feel that the invocation of marked non linearity between the animal response levels and the human exposures is somewhat misleading. I would feature both the linear and nonlinear approaches, with a fuller discussion of the ways in which each of these may be misleading if taken too literally. It is a shame that the risk characterization has not examined the attempt by Evans and coworkers (1994, Regul Toxicol Pharmacol 20:15-36) to create a distributional approach to chloroform potency assessment, based on expert judgment regarding the relative credence to be put on a variety of

analytical approaches, including linear and nonlinear, administered doses versus internal pharmacokinetic doses, cytotoxicity and genotoxicity, and other factors. This paper showed that no single approach suffices, but that a collection of approaches, each weighted by its judged likelihood to be appropriate, captures the full weight of evidence and span of possibilities.

I do feel that the document would substantially profit from more discussion of the pros and cons of various approaches and a more forthright discussion of how the Agency views each of the contentious and problematic issues. The document should show more explicitly what was done and should discuss more thoroughly why things were done in that way.

> **Response:** These comments were used to change the text in several ways in order to create an approximation of what the commentor intended, within the overall scope and intent of a risk characterization document.

R. Julian Preston, Ph.D.
Chemical Industry Institute of Toxicology

Comment:
As discussed, there are no data that indicate that chloroform induces tumors in humans. However, there is sufficient evidence showing that chloroform can induce tumors in rodents (rats and mice), but that the particular response can vary with route of exposure, administration vehicle, and strain of rodent. Thus, establishing which data set is the most appropriate one for use in estimating cancer risk to humans is not particularly easy. The report utilizes the incidence of renal tumors in male rats exposed to chloroform in drinking water (Jorgenson et al., 1985). The justification is not very clearly laid out, although it would appear to be an appropriate choice, especially absent a published report of the data from Matsushima et al. (1994).

> **Response:** Text was improved to justify the chosen study. Further justification can be found on EPA's IRIS (U.S. EPA, 1998).

Comment:
Although evidence for relevant effects in humans suggesting carcinogenic effects of chloroform is not strong, the fact that chloroform induces male liver tumors in female Wistar rats and kidney tumors in BDF1 male mice and Osborne-Mendel rats is suggestive of the potential for human carcinogenicity in a qualitative sense. At the same time the variability of response according to strain, especially absence in some strains, is worthy of somewhat more discussion in the report. In particular, there is a need to discuss the choice of data set for modeling more detail.

> **Response:** These points were further discussed in the text as indicated.

Comment:
The very weak genotoxicity or absence of genotoxicity argues quite effectively that the pathway to a tumor involves the indirect production of mutations, bearing in mind that mutations have to be formed by some mechanism in order that a tumor can develop via its multistep process.

Response: Text was enhanced to address this issue. Background mutations were suggested as one source of initiated cells.

Comment:
The discussion of mode of action in the report is generally clear and accurate. The rather close relationship between regenerative cell proliferation as a result of cytotoxicity and the induction of tumors suggests a link between the two. However, it needs to be made clearer that cell proliferation is perhaps a necessary step but clearly not a sufficient step by itself for tumor induction. Something else is needed, albeit weak genotoxicity, oxidative DNA damage, other secondarily produced DNA damages, or even an error-prone replication net result would seem to result in an overall dose response for tumors that is nonlinear and perhaps reflected by the curve for cell proliferation. This avoids the concern that is often expressed that tumors do not always arise when cell proliferation is increased (and perhaps also the corollary that tumors arise absent cell proliferation) by chloroform treatment.

Response: Text was added to clarify the distinction between the induction and the subsequent growth of tumors induced by the cell proliferation. Text was also added to further enhance the issue of oxidative DNA damage.

Comment:
This is a clearly written, concise report that follows a defined path to arrive at a risk assessment for human renal tumors from drinking water exposure. The choice of the rodent cancer data for the assessment needs to be justified more clearly. The mode of action needs to describe a process whereby tumors will result, given that cancer is a *genetic* disease. The Report successfully follows an approach consistent with EPA's 1996 Proposed Guidelines for Carcinogen Assessment.

Response: The choice and justification of rodent model was more clearly described; reference was made to EPA's IRIS where further support is shown. Statements were added to more clearly describe the cancer process as one that involves several steps, one of which is genetic damage.

Mode of Action SAB Review

(Letter from the Assistant Administrator of the Office of Water to the interim chair)

Dr. Morton Lippmann, Interim Chair
Science Advisory Board
Dr. Mark Utell, Co-Chair
Dr. Richard Bull, Co-Chair
Chloroform Risk Assessment Review Subcommittee
Science Advisory Board
U.S. Environmental Protection Agency
Ariel Rios Building
1200 Pennsylvania Avenue, N.W. (1400A)
Washington, DC 20460

Subject: Review of the EPA's Draft Chloroform Risk Assessment

Dear Dr. Lippmann, Dr. Utell, and Dr. Bull:

The Agency appreciates the efforts of the Science Advisory Board (SAB) in conducting a review of the cancer risk assessment for chloroform in October 1999. We requested a two-part review of the assessment. One part was to address issues about the Carcinogen Risk Assessment Guidelines. The second part was to address mode of action data and epidemiologic information.

The SAB reported its findings on the first part of the review questions on December 15, 1999, and a full report, including the second part, on April 28, 2000. The Agency appreciates these helpful and timely actions. This response is to the Report as it addresses the following questions as quoted in the Report:

> In the draft chloroform risk assessment document, are the conclusions as to the following issues adequately supported by the analyses presented in the health risk assessment/characterization (as supported by the ILSI report) and the framework analysis?
>
> 1) chloroform's mode of action
> 2) consideration of a nonlinear approach to dose-response, and the possibility that mutagenesis might play a role in the carcinogenic response.
> 3) the relationship of low-dose pathology to the doses that induce tumors
> 4) epidemiologic evidence on chlorinated drinking water as to the carcinogenicity of chloroform, including comment on any conclusion to be drawn from the epidemiologic data about mode of action.

Does the assessment of children's risk for chloroform appropriately address the risk concerns, including ontogeny of drug metabolizing enzymes, given the data available?

The Office of Water agrees with the recommendations in the report as to the chloroform assessment and is incorporating them in a new Toxicological Review that is being developed in support of the Agency's Integrated Risk Information System. The enclosed summary addresses the SAB's major overall findings and recommendation.

The Agency looks forward to further dialogue with the SAB as we move forward in our risk assessments supporting implementation of the Safe Drinking Water Act.

Sincerely,

J. Charles Fox
Assistant Administrator

Enclosure

Response to Science Advisory Board Recommendations

In October 1999 the Chloroform Risk Assessment Review Subcommittee of the Science Advisory Board met to consider the Office of Science and Technology health assessment of chloroform. Summaries of the major parts of the subcommittee's advice and our responses follow. The documents reviewed were a final hazard and dose-response characterization document and a draft mode-of-action framework analysis.

1. The Subcommittee agreed with EPA that sustained or repeated cytotoxicity with secondary regenerative hyperplasia in the liver and/or the kidney of rats and mice precedes, and is probably a causal factor for, hepatic and renal neoplasia. Some members of the subcommittee were concerned about possible mutagenic activity, and the subcommittee recommended that the risk assessment further address the possible role of mutagenicity as a mode of action.

The Office of Water will include a more complete analysis of mutagenic potential in its upcoming Toxicological Profile of Chloroform.

2. The subcommittee concluded that a nonlinear margin-of-exposure approach is scientifically reasonable for the liver tumor response because of the strong role cytotoxicity appears to play. In contrast, the application of the standard linear approach to the liver tumor data is likely to substantially overstate the low-dose risk. In addition, there is considerable question about this response because it is not produced when chloroform is administered to mice in drinking water.

   For the kidney response — because sustained cytotoxicity plays a clear role in the rat — a margin-of-exposure is a scientifically reasonable approach. Most members of the subcommittee thought that genotoxicity might possibly contribute to low-dose response in this organ, while some thought it unlikely.

The Office of Water will consider and address this possibility in the Toxicological Review in conjunction with discussions of metabolism and mutagenicity.

3. The subcommittee concluded that the extensive epidemiologic evidence relating drinking water disinfection (specifically chlorination) with cancer has little bearing on the determination of whether chloroform is a carcinogen. It added recommendations for discussion of endpoints and the potential meaning of these data to the assessment of chloroform.

The Office of Water notes that the hazard and dose-response assessment document reviewed did not contain the complete analysis of epidemiologic studies and the population-attributable risk analysis. The latter were separately provided to the subcommittee. The

opportunity will be taken to provide the recommended contextual discussion drawn from these documents to the Toxicological Review on Chloroform.

4. The subcommittee found that the draft mode-of-action document addressed children's risks quite adequately, based on the scientific information currently available. The major conclusions were believed correct, the role of CYP2E1 should be expressed as important, but its definitive role in the developing human or (other) mammal has yet to be confirmed. Nevertheless, the subcommittee report discussed several areas of the discussion of children's risk that can be improved, including exposure latency and transplacental and transmamillary exposure.

The Office of Water will address the needed improvements in its Toxicological Review. As the advice on some issues appears to be applicable beyond the chloroform assessment and carry implications for Agency guidance documents, the advice will be discussed with the EPA Risk Assessment Forum.

APPENDIX B
QUANTITATIVE DOSE-RESPONSE MODELING

B1: NON-CANCER BENCHMARK DOSE ANALYSIS
B2: CANCER DOSE-RESPONSE MODELING

(see attached electronic file modeling.zip)